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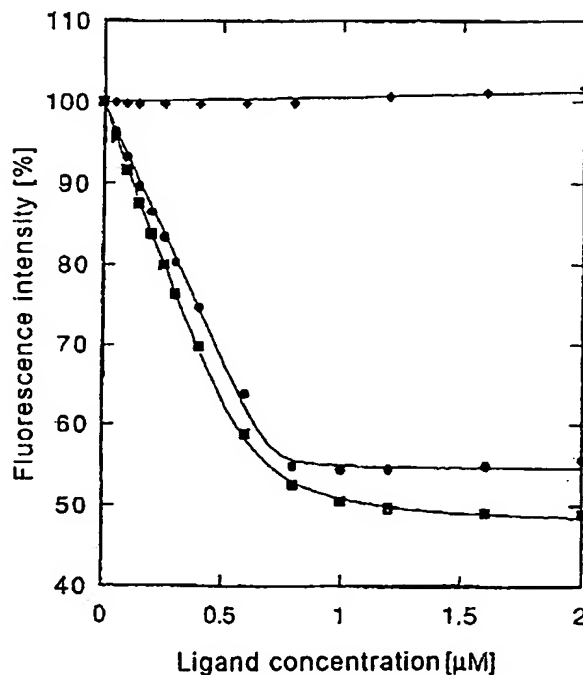
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(54) Titre : MUTEINES DE LA PROTEINE LIANT LA BILINE
(54) Title: MUTEINS OF BILIN-BINDING PROTEIN



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(57) Abrégé/Abstract

The invention relates to muteins of bilin-binding protein with a binding ability to digoxigenin and the fusion proteins of said muteins, a method for preparing said muteins and fusion proteins thereof and to their utilization for detecting or binding digoxigenin-labeled biomolecules. The invention especially relates to a polypeptide selected from the muteins of the bilin-binding protein, which is characterized in that (a) it can bind digoxigenin or digoxigenin conjugates; (b) it does not bind ouabain, testosterone and 4-aminofluorescein (c) at least one of the sequence positions 28, 31, 34, 35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125 and 127 of the bilin-binding protein has an aminoacid substitution. Due to their simple molecular structure, the inventive muteins provide advantages for production and utilization in comparison with antibodies against the digoxigenin group.

Abstract

The present invention relates to muteins of the bilin-binding protein with binding activity to digoxigenin
5 and to fusion proteins of such muteins, a method for preparing said muteins and fusion proteins thereof and their utilization for detecting or binding digoxigenin-labeled biomolecules. The invention especially relates to a polypeptide selected from muteins of the bilin-binding protein, characterized in that (a) it can bind
10 digoxigenin or digoxigenin conjugates, (b) it does not bind ouabain, testosterone, and 4-aminofluorescein and (c) at least one of the sequence positions 28, 31, 34, 35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125,
15 and 127 of the bilin-binding protein has an amino acid substitution. Due to their simple molecular structure, the inventive muteins provide advantages for production and utilization in comparison with antibodies against the digoxigenin group.

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Muteins of the bilin-binding protein

The present invention relates to muteins of the bilin-binding protein which are capable of binding digoxigenin and to fusion proteins of such muteins, to methods for preparing muteins of this kind and their fusion proteins and also to the use thereof for detecting or binding biomolecules labeled with digoxigenin.

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In molecular biology, the digoxigenin group is these days a very common instrument for nonradioactive detection of nucleic acids, proteins and other biomolecules. For this purpose, the biomolecule is, mostly covalently, modified with a reactive digoxigenin derivative, thus allowing subsequent detection of the molecule using an antibody directed against the digoxigenin group or a conjugate of an appropriate antibody fragment and a reporter enzyme, according to generally used methods in biochemistry.

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The skilled worker knows quite a number of reactive digoxigenin derivatives, which are in part also commercially available. For example, digoxigenin-3-O-methylcarbonyl- ϵ -aminocaproic acid N-hydroxysuccinimide ester (DIG-NHS), digoxigenin-3-O-succinyl- ϵ -aminocaproic acid N-hydroxysuccinimide ester and 3-amino-3-deoxydigoxigenin-hemisuccinimide succinimidyl ester are suitable for covalent coupling to proteins, in particular to the amino groups of exposed lysine side chains. Using 3-iodoacetyl-amino-3-deoxydigoxigenin it is possible to label especially thiol groups in proteins or in other biomolecules in a selective manner with the digoxigenin group. It is possible to couple synthetic oligodeoxynucleotides to the same reactive digoxigenin derivatives, as long as they have been equipped with suitable free amino or thiol groups

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In addition, cis-platinum complexes of digoxigenin derivatives (DIG Chem-Link reagent) or digoxigenin derivatives containing carbodiimide groups (disclosed
5 in the European patent specification EP 0 806 431 A2) are suitable for the direct labeling of nucleic acids. Alternatively, it is possible in the case of deoxyribonucleic acids to label said deoxyribonucleic acids during a template-dependent enzymatic synthesis
10 with the aid of a DNA polymerase and a deoxynucleotide triphosphate coupled to the digoxigenin group, for example digoxigenin-11-dUTP, digoxigenin-11-ddUTP or digoxigenin-16-dATP. Analogously, digoxigenin-11-UTP is suitable for incorporation into enzymatically
15 synthesized RNA. Moreover, it is possible to label oligodeoxynucleotides with the digoxigenin group directly in the automated DNA synthesis by using suitable activated building blocks, for example so-called "virtual nucleotides". Digoxigenin group-coupled
20 nucleic acids of this kind are suitable as nonradioactive gene probes for detection of complementary nucleotide sequences by hybridization, for example in Northern or Southern blots (disclosed in the European patent specification EP 0 324 474 A1).

25 Digoxigenin group-labeled proteins or glycoproteins are particularly useful for determining, for example, relevant antigens or antibodies directed there against in immunochemical assay methods such as ELISA (enzyme-linked immunosorbent assay). The biomolecule conjugated
30 with the digoxigenin group is actually detected using an anti-digoxigenin antibody, normally in the form of a conjugate of the Fab fragment of said antibody with a suitable enzyme, such as, for example, alkaline
35 phosphatase or horseradish peroxidase, as label. The enzymatic activity then serves for quantification via catalysis of a chromogenic, fluorogenic or chemiluminescent reaction. Various antibodies against

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J. Immunol. 129 (1982), 1165-1172; Jeffrey et al., J. Mol. Biol. 248 (1995), 344-360).

The use of antibodies, however, has several
5 disadvantages. Hence, the preparation of monoclonal
antibodies in hybridoma cell cultures is complicated,
and the proteolysis for the Fab fragment and also the
production of conjugates with reporter enzymes requires
additional difficult processing steps. But even the
10 production of antibodies by genetic engineering is not
simple, and the main reason for this is that antibodies
as well as their antigen-binding fragments are composed
of two different polypeptide chains in a structurally
complicated manner. For the genetic manipulation of
15 antibodies it is therefore necessary to handle two
genes simultaneously. Moreover, the yield of correctly
folded antibody fragments produced by genetic
engineering is often low. As is known to the skilled
worker, this is even more so when recombinant fusion
20 proteins are to be prepared from Fab fragments of
antibodies and enzymes.

It was therefore the object of the invention to develop
alternative polypeptide reagents for detection of the
25 digoxigenin group, which can be produced in a simple
manner.

In an evolutionary research approach, it has
surprisingly been found now that muteins of the bilin-
30 binding protein, which is structurally based on a
single polypeptide chain (Schmidt and Skerra, Eur. J.
Biochem. 219 (1994), 855-863), are suitable for
detecting the digoxigenin group by binding with high
affinity, whereby the recognition of digoxigenin is
35 astoundingly selective compared with other steroids.

The present invention thus relates to a polypeptide,
selected from muteins of the bilin-binding protein,

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(a) is able to bind digoxigenin or digoxigenin conjugates.

(b) does not bind ouabain, testosterone, and 4-aminofluorescein and

5 (c) has an amino acid substitution at at least one of the sequence positions 28, 31, 34, 35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125, and 127 of the bilin-binding protein.

10 In this connection, preference is given to digoxigenin-binding muteins which have an amino acid substitution at at least 4 to 7 or, preferably, at least 8 to 12 of the sequence positions defined above. A particularly preferred mutein is the polypeptide which has the amino
15 acid sequence depicted as SEQ ID NO. 15.

Outside the region of the amino acid positions 28, 31, 34, 35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125, and 127 the muteins of the present invention may
20 correspond to the amino acid sequence of the bilin-binding protein from *Pieris brassicae*. On the other hand, the amino acid sequence of the polypeptides of the invention may also have differences to the bilin-binding protein apart from said positions. Bilin-
25 binding protein sequence variants of this kind comprise naturally occurring and also artificially generated variants, and the deviations mean substitutions, insertions, deletions of amino acid residues and also N- and/or C-terminal additions.

30

For example, the inventive muteins of the bilin-binding protein may have amino acid substitutions which prevent oligomerization of the bilin-binding protein, such as the Asn(1)->Asp substitution, or suppress proteolytic

35 cleavage within the polypeptide chain, which may occur during production in *E. coli*, such as, for example, by the Lys(87)->Ser substitution. Furthermore, the mutations Asn(21)->Gln and Lys(135)->Met may be

of the bilin-binding protein, in order to facilitate, for example, cloning of a gene segment via two new BstXI restriction cleavage sites at these positions. Likewise, the present invention relates to the specific introduction of amino acid substitutions within or outside the said positions, in order to generally improve particular properties of the mutein of the invention, for example its folding stability or folding efficiency or its resistance to proteases.

10

The ability of the polypeptides of the invention to bind digoxigenin or digoxigenin conjugates can be determined by common methods, for example ELISA, fluorescence titration, titration calorimetry, surface plasmon resonance measurements or blotting methods, for example Western blotting, Southern blotting or Northern blotting. Blotting methods may be used in order to transfer conjugates of digoxigenin with proteins or nucleic acids to a membrane and then detect said conjugates using one of the muteins of the invention, a conjugate of this mutein or a fusion protein of this mutein.

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A quantitative parameter for binding affinity is provided by established thermodynamic parameters such as, for example, the affinity constant or dissociation constant for the complex of mutein and bound ligand, for example digoxigenin. However, it is also possible to determine the binding ability qualitatively, for example based on the intensity of a binding signal due to a chromogenic reaction or of a colored precipitate which is obtained with the aid of one of said blotting methods.

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Preferred muteins of the invention are obtainable in a two-stage evolutionary process. Random mutagenesis of the bilin-binding protein at at least one, preferably at at least 4 to 7, and particularly preferably at at

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35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125, and 127 and subsequent simple or, preferably, repeated selection of muteins with affinity for the digoxigenin group from this library, preferably using free
5 digoxigenin or digitoxigenin for competitive enrichment, provides muteins of the bilin-binding protein which recognize the digoxigenin group, but the affinity is still comparatively low. Renewed mutagenesis of such a mutein at at least one,
10 preferably at at least 3 or 4, or at all of amino acid positions 28, 31, 34, 35, 36 and 37, now followed by a simple or, preferably, repeated enrichment by formation of a complex with the digoxigenin group and by subsequent dissociation of the formed complex in an
15 acidic or basic milieu, then results in obtaining muteins having substantially higher affinity for the digoxigenin group. The digoxigenin group is preferably present as a digoxigenin/biotin double conjugate during said enrichment.

20 Surprisingly, it has now been found that the affinity constant between such polypeptides of the invention and digoxigenin is at least 10^7 M^{-1} . This means in other words that the dissociation constant of the complex
25 between the polypeptide of the invention and digoxigenin is 100 nM or less. Individual species even show dissociation constants of 35 nM or less, as illustrated in the Examples.

30 Besides digoxigenin, the inventive muteins of the bilin-binding protein can also bind digoxigenin derivatives as ligands, for example digoxin, digitoxin or digitoxigenin. Furthermore, the inventive muteins of the bilin-binding protein may bind conjugates of said
35 chemical compounds, i.e. nucleic acids, polypeptides, carbohydrates, other natural or synthetic biomolecules, macromolecules or low molecular weight compounds which are covalently linked or linked via a metal complex to

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Preference is given to using for the preparation of such conjugates the reactive derivatives of digoxigenin, digoxin, digitoxin or digitoxigenin, which are known to the skilled worker and are stated, for example, further above.

Preferred muteins of the invention, which were obtained by the two-stage process described, show, compared with the affinity for digoxigenin, an even higher affinity for digitoxin or digitoxigenin, whose steroid system differs from that of digoxigenin only by the absence of a hydroxyl group. Surprisingly, these muteins show distinctive specificity with respect to the digoxigenin or digitoxigenin group, and this is shown by the fact that other steroids or steroid groups such as ouabain or testosterone are bound with much less affinity, if at all. Fluorescein derivatives such as 4-amino-fluorescein, too, are evidently not bound. This means that ouabain, testosterone or 4-aminofluorescein in each case exhibit a dissociation constant of at least 10 μ M, preferably at least 100 μ M, with respect to the inventive muteins of the bilin-binding protein.

This property of specificity distinguishes said muteins considerably from other muteins of the bilin-binding protein and also from antibodies directed against the digoxigenin group, such as, for example, the antibody 26-10 (Chen et al., Protein Eng. 12 (1999), 349-356), which binds ouabain with substantial affinity, and gives the inventive muteins of the bilin-binding protein a particular advantage. It is surprising that particularly the additional amino acid substitutions at positions 28, 31, 34, 35, 36, and 37 lead to the preferred muteins of the bilin-binding protein.

Preference is therefore given to those muteins which carry at least one, preferably at least 3 or 4, or all of the amino acid substitutions Glu(28)->Gln, Lys(31)->Ala, Asn(34)->Asp, Ser(35)->His, Val(36)->Ile

Particularly preferred muteins of the invention carry, when compared to the bilin-binding protein, at least one, at least 4 to 7, or, preferably, at least 8 to 12 of the amino acid substitutions selected from Glu(28)->Gln, Lys(31)->Ala, Asn(34)->Asp, Ser(35)->His, Val(36)->Ile, Glu(37)->Thr, Asn(58)->Arg, His(60)->Ser, Ile(69)->Ser, Leu(88)->Tyr, Tyr(90)->Ile, Lys(95)->Gln, Asn(97)->Gly, Tyr(114)->Phe, Lys(116)->Ser, Gln(125)->Met and Phe(127)->Leu. The representation chosen indicates in each case first the amino acid in the natural bilin-binding protein (SWISS-PROT database accession code P09464) together with the sequence position for the mature polypeptide in brackets, and the corresponding amino acid in a mutein of the invention is stated after the arrow. Very particularly preferred muteins according to this invention carry all of the amino acid substitutions mentioned.

Surprisingly, position 93 of the bilin-binding protein is unchanged in the muteins of the invention, although this amino acid, too, had been affected by the mutagenesis for preparing the random library. Preferred muteins of the bilin-binding protein therefore carry the amino acid Val at said position.

It is an advantage for particular detection methods to use the muteins of the bilin-binding protein of the present invention in a labeled form. Accordingly, this invention further relates to a polypeptide of the invention which is characterized in that it carries at least one label. Suitable labeling groups are known to the skilled worker and include enzyme label, radioactive label, fluorescent label, chromophoric label, (bio)luminescent label or a label containing haptens, biotin, metal complexes, metals or colloidal gold. Very generally, labeling is possible with substances or enzymes which generate a determinable

connection it is possible to couple all known labels for antibodies to the muteins of the invention, too.

A possibility which is particularly advantageous for practical application is to use the inventive muteins of the bilin-binding protein in the form of fusion proteins. Techniques for preparing such fusion proteins by means of genetic engineering methods are known to the skilled worker. Suitable fusion partners for the muteins of the invention would be enzymes and other polypeptides, proteins or protein domains. Such fusions would be suitable for providing the mutein of the bilin-binding protein with additional properties such as, for example, enzymatic activity or affinity for other molecules, such as proteins, macromolecules or low molecular weight ligands.

For example, fusions are possible with enzymes which catalyze chromogenic or fluorogenic reactions or may serve for the release of cytotoxic agents. Further examples for fusion partners which may be advantageous in practice are binding domains such as the albumin-binding domain or the immunoglobulin-binding domain of protein G or protein A, antibody fragments, oligomerization domains, toxins or other binding proteins and functional parts thereof and also affinity peptides such as, for example, the Strep-tag or the Strep-tag II (Schmidt et al., J. Mol. Biol. 255 (1996), 753-766). Suitable fusion partners are also proteins having particular chromogenic or fluorogenic properties, such as, for example, green fluorescent protein. Another suitable fusion partner would be the coat protein III of a filamentous bacteriophage, such as M13, f1 or fd, or a fragment of said coat protein.

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Very generally, the term fusion protein is intended here to mean also those inventive muteins of the bilin-binding protein, which are equipped with a signal

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polypeptide of the invention may serve for the purpose of directing said polypeptide during biosynthesis into a particular cell compartment, for example the *E. coli* periplasm or the lumen of the endoplasmic reticulum of a eukaryotic cell, or into the medium surrounding the cell. The signal sequence is typically cleaved off by a signal peptidase. In addition, it is also possible to use other signal or targeting sequences which need not necessarily be located at the N-terminus of the polypeptide and which make it possible to locate said polypeptide in specific cell compartments. A preferred signal sequence for secretion into the *E. coli* periplasm is the OmpA signal sequence. A large number of further signal sequences and also targeting sequences are known in the prior art.

An advantage of the inventive muteins of the bilin-binding protein is the suitability of both their N-terminus and their C-terminus for preparing fusion proteins. In contrast to antibodies, in which the N-terminus of both the light and the heavy immunoglobulin chain are in spatial proximity to the antigen binding site, it is possible to use in the polypeptides of the invention both ends of the polypeptide chain for the preparation of fusion proteins, without adversely affecting ligand binding.

The invention therefore also relates to fusion proteins of muteins of the bilin-binding protein in which an enzyme, another protein or a protein domain, a signal sequence and/or an affinity peptide is fused to the amino terminus of the polypeptide in an operable manner. The invention yet further relates to fusion proteins of bilin-binding protein muteins or of fusion proteins with the amino terminus of bilin-binding protein muteins in which an enzyme, another protein or a protein domain, a targeting sequence and/or an affinity peptide is fused to the carboxy terminus of

A preferred enzyme for constructing the fusion proteins of the invention is bacterial alkaline phosphatase (Sowadski et al., J. Mol. Biol. 186 (1985) 417-433), which may be attached either at the N-terminus or at the C-terminus of a mutein of the bilin-binding protein. In addition, such a fusion protein may carry a signal sequence such as, for example, OmpA or PhoA, which effects secretion of said fusion protein into the *E. coli* periplasm, where the disulfide bonds of the polypeptide chain may form efficiently. Furthermore, it may be equipped with an affinity peptide such as, for example, the Strep-tag II, which allows easy purification of said fusion protein. Specific fusion proteins of the invention are described in the Examples. An advantage of a fusion protein of this kind is its ability to catalyze directly a chromogenic, fluorogenic or chemiluminescent detection reaction, which simplifies its use for detection of the digoxigenin group.

Another advantage of using alkaline phosphatase for constructing fusion proteins of the invention is the fact that this enzyme forms a stable homodimer and, consequently, confers the property of bivalence on the bilin-binding protein mutein as part of the fusion protein. In this way, binding of the digoxigenin group may result in an avidity effect, which increases detection sensitivity. Such an avidity effect can be expected in particular if the digoxigenin-labeled molecule is adsorbed to a solid phase, is present in oligomeric or membrane-bound form or is conjugated with a plurality of digoxigenin groups. Analogously, other homodimeric enzymes are suitable for preparing bivalent fusion proteins containing the inventive muteins of the bilin-binding protein.

Apart from bacterial alkaline phosphatase, it is also

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such as, for example, calf intestine phosphatase (CIP), for preparing fusion proteins of the invention. Said phosphatases are frequently distinguished by higher enzymatic activity (Murphy and Kantrowitz, Mol. Microbiol. 12 (1994), 351-357), which may result in higher detection sensitivity. It is also possible to use mutants of bacterial alkaline phosphatase, which have improved catalytic activity (Mandecki et al., Protein Eng. 4 (1991), 801-804), for constructing fusion proteins of the invention. Other enzymes known to the skilled worker which catalyze chromogenic, fluorogenic or chemiluminescent reactions, such as, for example, β -galactosidase or horseradish peroxidase, are also suitable for preparing fusion proteins of the invention. Moreover, all these enzymes may likewise be employed for labeling muteins of the bilin-binding protein by conjugating them, for example by using common coupling reagents, with the separately obtained mutein or a fusion protein of the mutein.

In another aspect, the present invention relates to a nucleic acid which comprises a sequence coding for a mutein or a fusion protein of a mutein of the bilin-binding protein. This nucleic acid may be part of a vector on which an operatively functional environment for expressing the nucleic acid is present. A large number of suitable vectors is known from the prior art and is not described in detail here. An operatively functional environment means those elements which allow, assist, facilitate and/or increase transcription and/or subsequent processing of an mRNA. Examples of elements of this kind include promoters, enhancers, transcription initiation sites, and transcription termination sites, translation initiation sites, polyadenylation signals, etc. In a preferred embodiment, such nucleic acids of the invention comprise a nucleic acid sequence which encodes the polypeptide sequence depicted as SEQ ID NO:15. Owing to

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skilled worker that the nucleotide sequence stated as SEQ ID NO:15 represents only a single nucleotide sequence from the group of nucleotide sequences encoding the polypeptide according to SEQ ID NO:15.

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The nucleic acid of the invention or its environment may be designed such that biosynthesis of the polypeptide takes place in the cytosol, in which case the polypeptide sequence being preceded, where appropriate, by a start methionine. In a preferred embodiment, however, an N-terminal signal sequence is used, in particular the OmpA or PhoA signal sequence, in order to direct the polypeptide of the invention into the *E. coli* periplasm, where the signal sequence is cleaved off by the signal peptidase and the polypeptide chain is able to fold with oxidative formation of the disulfide bonds. Eukaryotic signal sequences may be used in order to secrete the polypeptide of the invention in a eukaryotic host organism.

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In principle, both prokaryotic, preferably *E. coli*, and eukaryotic cells such as, for example, yeasts are considered for expression of the nucleic acid of the invention.

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In yet another aspect, the present invention relates to a method for preparing an inventive mutein or fusion protein of a mutein of the bilin-binding protein, which method is characterized in that the nucleic acid coding for the mutein or the fusion protein of a mutein of the bilin-binding protein is expressed in a bacterial or eukaryotic host cell and the polypeptide is obtained from the cell or the culture supernatant. For this purpose, normally a suitable host cell is first transformed with a vector which comprises a nucleic acid coding for a polypeptide of the invention. The host cell is then cultured under conditions under which

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bio-synthesis of the polypeptide occurs, and the polypeptide of the invention is obtained.

With respect to the preparation method, it must be taken into account that the inventive muteins of the bilin-binding protein have two structural disulfide bonds and that additional disulfide bonds may be present in corresponding fusion proteins. The formation of said disulfide bonds, which takes place during protein folding, is normally ensured if the polypeptide of the invention is directed with the aid of a suitable signal sequence into a cell compartment containing an oxidizing thiol/disulfide redox milieu, for example into the bacterial periplasm or the lumen of the endoplasmic reticulum of a eukaryotic cell. In this respect, the polypeptide of the invention can be liberated by cell fractionation or obtained from the culture supernatant. It is possible, where appropriate, to increase the folding efficiency by overproducing protein disulfide isomerases, for example *E. coli* DsbC protein, or proteins that assist folding.

On the other hand, it is possible to produce a polypeptide of the invention in the cytosol of a host cell, preferably *E. coli*. The said polypeptide may then be obtained, for example, in the form of inclusion bodies and afterwards be renatured *in vitro*. Depending on the intended use, the protein can be purified by means of various methods known to the skilled worker. A suitable method for purifying the inventive muteins of the bilin-binding protein is, for example, affinity chromatography using a column material which carries digoxigenin groups. In order to purify fusion proteins of the muteins of the bilin-binding protein, it is possible to utilize the affinity properties of the fusion protein, which are known from the prior art, for example those of the Strep-tag or the Strep-tag II (Schmidt and Skerra, J. Chromatogr. A 676 (1994), 337-

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those of the albumin binding domain (Nygren et al., J. Mol. Recogn. 1 (1988), 69-74) or those of alkaline phosphatase (McCafferty et al., Protein Eng. 4 (1991) 955-961). The fact that the muteins of the bilin-binding protein consist only of a single polypeptide chain is advantageous for the methods for preparing the polypeptides of the invention, since no care needs to be taken either of synthesizing several different polypeptide chains within a cell simultaneously or of different polypeptide chains associating with one another in a functional manner.

The possibilities for practical application of the inventive muteins of the bilin-binding protein essentially correspond to those for conventional antibodies or antibody fragments with binding affinity for digoxigenin. Accordingly, the invention also relates to the use of a mutein of the invention or of a fusion protein of a mutein of the bilin-binding protein in a method for detecting, determining, immobilizing or removing digoxigenin or conjugates of digoxigenin with proteins, nucleic acids, carbohydrates, other biological or synthetic macromolecules or low molecular weight chemical compounds.

The inventive muteins of the bilin-binding protein or their fusion proteins can be used in detection methods essentially in a manner analogous to corresponding detection methods known for anti-digoxigenin antibodies and also fragments and/or conjugates thereof. In a further aspect, the present invention therefore relates to a method for detecting the digoxigenin group, in which method a mutein of the bilin-binding protein or a fusion protein of a mutein of the bilin-binding protein is brought into contact with digoxigenin or with digoxigenin conjugates under conditions suitable for effecting binding of the mutein to the digoxigenin group and the mutein or the fusion protein of the

For this purpose, the mutein may be labeled directly, for example by covalent coupling. It is, however, also possible to use indirect labeling, for example by means of labeled antibodies against the bilin-binding protein or muteins thereof or against domains of fusion proteins of these muteins. The use of inventive fusion proteins containing an enzyme, for example alkaline phosphatase, instead of a labeled mutein of the bilin-binding protein is particularly advantageous. In this case, it is possible to design the determination method with a particularly small number of process steps, whereby, for example, the ability of the enzyme as part of the fusion protein to catalyze a chromogenic, fluorogenic or luminescent detection reaction may be directly utilized. Here, the fact that such fusion proteins are readily available is a particular advantage compared with corresponding fusion proteins of conventional antibodies. Utilization of the above-described avidity effect in the case of an oligomeric fusion protein is a further advantage in such a method.

It is possible to carry out a method for determining the digoxigenin group, for example, for qualitatively detecting nucleic acids conjugated with the digoxigenin group in Southern or Northern blots or proteins conjugated with the digoxigenin group in Western blots. A determination method may also be carried out quantitatively for detecting proteins conjugated with the digoxigenin group in an ELISA. In addition, a determination method of the invention is also suitable for indirect detection of proteins not conjugated with digoxigenin or of other molecules by using a binding protein which is directed against the protein or molecule, for example an antibody or its fragment, and which is conjugated with the digoxigenin group. Indirect detection of the nucleic acids not conjugated with digoxigenin is also possible by using a gene probe

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conjugated with the digoxigenin group. An application in medical diagnostics or therapy results furthermore from the determination of digoxigenin, digoxin, digitoxin or digitoxigenin, without these ligands
5 having to be conjugated with another molecule.

The muteins of the invention or fusion proteins thereof may also be used for immobilizing a molecule conjugated with the digoxigenin group. This immobilization is
10 preferably carried out on solid phases coated with the muteins or their fusion proteins, such as, for example, microtiter plates, immunosticks, microbeads made of organic, inorganic or paramagnetic materials, or sensor surfaces.

15 Correspondingly, it is likewise possible to use the muteins of the invention or fusion proteins thereof for removing digoxigenin, digoxin, digitoxin or digitoxigenin, or a molecule conjugated with one of
20 these compounds. In this case, in addition to the solid phases mentioned, column materials are also considered for coating with the muteins or their fusion proteins. Preferably, said coating is carried out on suitable column materials by coupling by means of chemically
25 reactive groups. Column materials coated in this way may be used for removing from a solution substances conjugated with digoxigenin groups and also, where appropriate, complexes of such substances with other molecules.

30 Thus, it is possible, for example, to remove antigens from a solution by adding to the solution antibodies which are directed against the antigens and are conjugated with the digoxigenin group, and contacting

35 the resulting solution with said column material under conditions under which complex formation between the digoxigenin groups and an inventive mutein of the bilin-binding protein or its fusion protein occurs.

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appropriate, to elute the substance conjugated with the digoxigenin. This elution may be carried out by competition with digoxin, digoxigenin, digitoxin or digitoxigenin and also, for example, by lowering or
5 increasing the pH of the solution. In a competitive elution it is possible to utilize in an advantageous manner the higher binding affinity of the muteins of the invention for digitoxigenin or digitoxin compared with the digoxigenin group. In this way it is possible
10 to isolate or purify a substance conjugated with digoxigenin.

The invention is further illustrated by the following Examples and attached drawings, in which:

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Figure 1 represents in each case a fluorescence titration of the Strep-tag II-fused mutein DigA16 with the ligands digoxigenin, digitoxigenin, and ouabain;

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Figure 2 depicts diagrammatically the expression vectors pBBP27 (A) and pBBP29 (B) for preparing fusion proteins of mutein DigA16 with alkaline phosphatase;

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Figure 3 demonstrates quantitative detection of biomolecules conjugated with digoxigenin groups by fusion proteins of mutein DigA16 with alkaline phosphatase in an ELISA;

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Figure 4 shows qualitative detection of biomolecules conjugated with digoxigenin groups by fusion proteins of mutein DigA16 with alkaline phosphatase on a Western blot.

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Figure 1 shows the graphic representation of results from Example 3 in which different concentrations of the steroids digoxigenin (squares), digitoxigenin (circles)

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of mutein DigA16. The particular protein fluorescence intensities were measured at an excitation wavelength of 295 nm and an emission wavelength of 345 nm and plotted as a function of the actual total steroid
5 concentration in the particular reaction mixture. Finally, the data points were fitted to a regression curve by means of non-linear regression.

Figure 2 shows a drawing of the expression vectors
10 pBBP27 (A) and pBBP29 (B). pBBP27 codes for a fusion protein of bacterial alkaline phosphatase with its own signal sequence, a peptide linker having the sequence Pro-Pro-Ser-Ala, the mutein DigA16 and also the Strep-tag II affinity tag. The corresponding structural gene
15 is followed by the *dsbC* structural gene (including its ribosomal binding site) from *E. coli* (Zapun et al., Biochemistry 34 (1995), 5075-5089) as second cistron. The artificial operon formed in this way is under joint transcriptional control of the tetracycline
20 promoter/operator ($tet^{p/o}$) and ends at the lipoprotein transcription terminator (t_{lpp}). Further vector elements are the origin of replication (*ori*), the intergenic region of filamentous bacteriophage f1 (*f1-IG*), the ampicillin resistance gene (*bla*) coding for β -lactamase
25 and the tetracycline repressor gene (*tetR*). pBBP29 codes for a fusion protein of the OmpA signal sequence, the mutein DigA16, the Strep-tag II affinity tag, a peptide linker consisting of five glycine residues, and bacterial alkaline phosphatase without its N-terminal
30 amino acid arginine. The vector elements outside this region are identical to vector pBBP27.

Figure 3 shows a graphic representation of the data
from Example 4 in which digoxigenin groups were
35 detected quantitatively with the aid of mutein DigA16 fusion proteins as gene products of vectors pBBP27 (closed symbols) and pBBP29 (open symbols). Here, the digoxigenin groups were coupled on the one hand to

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hand, to chicken egg albumin (ovalbumin, triangles). The control data shown are those obtained when using underivatized bovine serum albumin and the fusion protein encoded by pBBP27 (open circles). The enzymatic activity corresponding to the particular bound fusion protein was monitored spectrophotometrically at 405 nm on the basis of p-nitrophenyl phosphate hydrolysis. Curve fitting was carried out by non-linear regression with the aid of the Kaleidagraph computer program (Abelbeck Software) by means of the equation

$$[P \cdot L] = [L]_t [P]_t / (K_d + [P]_t).$$

Here, $[P]_t$ corresponds to the total fusion protein concentration used in the particular microtiter plate well. $[P \cdot L]$ is determined on the basis of the enzymatic activity of alkaline phosphatase. The total concentration of digoxigenin groups $[L]_t$, constant within a concentration series, per well and the dissociation constant K_d were fitted as parameters by non-linear regression.

Figure 4 shows the result of a Western blot experiment from Example 4 for qualitative detection of biomolecules conjugated with digoxigenin groups by means of the mutein DigA16 fusion proteins encoded by pBBP27 (lanes 1 and 2) and pBBP29 (lanes 3 and 4). For comparison, a 15% strength SDS polyacrylamide gel of the biomolecules, stained with Coomassie Brilliant Blue, is also shown (lanes 5 and 6). Here, a mixture of 0.5 μ g of underivatized BSA, underivatized ovalbumin and underivatized RNaseA was fractionated in each case in lanes 1, 3 and 5. A mixture of 0.5 μ g of BSA coupled to digoxigenin groups, ovalbumin coupled to digoxigenin groups and RNaseA coupled to digoxigenin groups was fractionated in each case in lanes 2, 4 and 6.

Examples

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Unless stated otherwise, the genetic engineering methods familiar to the skilled worker, as described, for example, in Sambrook et al. (Molecular Cloning. A Laboratory Manual (1989), Cold Spring Harbor Press) were used.

Example 1: Preparation of a library for muteins of the bilin-binding protein, phagemid presentation and selection of a mutein with binding affinity for digoxigenin

A library for muteins of the bilin-binding protein was prepared by subjecting the amino acid sequence positions 34, 35, 36, 37, 58, 60, 69, 88, 90, 93, 95, 97, 114, 116, 125 and 127 of the bilin-binding protein to a concerted mutagenesis in multiple steps by means of the polymerase chain reaction (PCR). The PCR reactions were initially carried out in two separate amplification steps in a volume of in each case 50 μ l, and 10 ng of pBBP20 phasmid DNA (SEQ ID NO:1) as template using in each case 25 pmol of two primers (SEQ ID NO:2 and SEQ ID NO:3 in one mixture and SEQ ID NO:4 and SEQ ID NO:5 in a second mixture) which had been synthesized according to the generally known phosphoramidite method were used.

Furthermore, the reaction mixture contained 5 μ l of 10xTaq buffer (100 mM Tris/HCl pH 9.0, 500 mM KCl, 1% v/v Triton X-100), 3 μ l of 25 mM MgCl₂ and 4 μ l of dNTP mix (2.5 mM dATP, dCTP, dGTP, dTTP). After filling up with water, the mixture was overlaid with mineral oil and heated to 94°C in a programmable thermostating block for 2 min. Then 2.5 u of Taq DNA polymerase (5 u/ μ l, Promega) were added and 20 temperature cycles of 1 min at 94°C, 1 min at 60°C and 1.5 min at 72°C were carried out, followed by an incubation at 60°C for 5 min. The desired amplification products were isolated via preparative agarose gel electrophoresis from low

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DNA extraction kit (Genomed) according to the manufacturer's instructions.

A relevant section of the pBBP20 nucleic acid sequence is shown together with the encoded amino acid sequence as SEQ ID NO:1 in the sequence listing. The section starts with a hexanucleotide sequence which was obtained by ligating an *Xba*I overhang with an *Spe*I overhang complementary thereto and ends with the *Hind*III cleavage site. The vector elements outside this region are identical to vector pASK75 whose complete nucleotide sequence is stated in the German Offenlegungsschrift DE 44 17 598 A1.

The subsequent amplification step was carried out in a 100 μ l mixture, and in each case approx. 6 ng of the two isolated fragments as template using 50 pmol of each of the two primers SEQ ID NO:6 and SEQ ID NO:7 and also 1 pmol of oligodeoxynucleotide SEQ ID NO:8. The remaining components of the PCR mixture were added in twice the amount, as in the preceding amplification steps. The PCR was carried out in 20 temperature cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C, followed by a final incubation at 60°C for 5 min. The fragment obtained was again isolated by preparative agarose gel electrophoresis.

For cloning this fragment which represented the mutein library in the form of a mixture of nucleic acids it was first cut with the restriction enzyme *Bst*XI (New England Biolabs) according to the manufacturer's instructions. The nucleic acid fragment obtained (335 base pairs, bp) was purified again by means of preparative agarose gel electrophoresis. Analogously, pBBP20 vector DNA was cut with *Bst*XI and the larger of the two fragments (3971 bp) was isolated.

For ligation, 0.93 μ g (4.2 pmol) of the PCR fragment

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incubated in the presence of 102 Weiss units of T4 DNA ligase (New England Biolabs) in a total volume of 500 μ l (50 mM Tris/HCl pH 7.8, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 50 μ g/ml BSA) at 16°C for two days. The DNA
5 was then precipitated by adding 10 μ g of yeast tRNA (Boehringer Mannheim), 25 μ l of 5 M ammonium acetate and 100 μ l of ethanol to in each case 24 μ l of the ligation mixture. Incubation at -20°C for 3 days was followed by centrifugation (25 min, 16000 g, 4°C). The
10 precipitate was washed in each case with 200 μ l of ethanol (70% v/v, -20°C) and dried under vacuum. Finally, the DNA was taken up in 43.6 μ l of TE/10 (1 mM Tris/HCl pH 8.0, 0.1 mM EDTA). The DNA concentration of the solution obtained was estimated by analytical
15 agarose gel electrophoresis on the basis of the fluorescence intensity of the bands stained with ethidium bromide in comparison with a DNA size standard of known concentration.

20 Preparation of electrocompetent cells of the *E. coli* K12 strain XL1-Blue (Bullock et al., BioTechniques 5 (1987), 376-379) was carried out according to the methods described by Tung and Chow (Trends Genet. 11 (1995), 128-129) and by Hengen (Trends Biochem. Sci. 21
25 (1996), 75-76). 1 l of LB medium was adjusted to an optical density at 600 nm, OD₆₀₀ = 0.08 by adding a stationary XL1-Blue overnight culture and incubated in a 3 l Erlenmeyer flask at 200 rpm and 26°C. After reaching OD₆₀₀ = 0.6, the culture was cooled on ice for
30 30 min and then centrifuged at 4000 g and 4°C for 15 min. The cell sediment was washed twice with in each case 500 ml of ice cold 10% w/v glycerol and finally resuspended in 2 ml of ice cold GYT medium (10% w/v glycerol, 0.125% w/v yeast extract, 0.25% w/v
35 tryptone).

Electroporation was carried out by using the Easyjec T Basic system (EquiBio) with the corresponding cuvettes

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carried out in a cold room at 4°C. 5 to 6 μ l of the above-described DNA solution (245 ng/ μ l) were in each case mixed with 40 μ l of the cell suspension, incubated on ice for 1 min and then transferred into the cuvette.

5 After electroporation, the suspension was immediately diluted in 2 ml of fresh ice-cold SOC medium (2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 10 mM MgSO₄, 10 mM MgCl₂) and agitated at 37°C and 200 rpm for 60 min. The cells were then sedimented at 3600 g for in

10 each case 2 min, resuspended in 1 ml of LB medium containing 100 μ g/ml of ampicillin (LB/Amp) and plated out at 200 μ l each on agar plates (140 mm in diameter) with LB/Amp medium. Using a total of 10.7 μ g of the ligated DNA in eight electroporation mixtures produced

15 in this way $3.73 \cdot 10^8$ transformants which were distributed on 40 agar plates.

After incubation at 32°C for 14 h, the colonies obtained in this way were scraped off the agar plates

20 with the addition of in each case 10 ml of 2xYT/Amp medium, transferred to a sterile Erlenmeyer flask and agitated at 37°C, 200 rpm for 20 min to complete resuspension. 50 ml of 2xYT/Amp medium prewarmed to 37°C were inoculated with 2.88 ml of said suspension so

25 that the cell density was 1.0 OD₅₅₀. This culture was incubated at 37°C, 160 rpm for 6 h to reach a stationary cell density, and phasmid DNA was isolated with the aid of the plasmid Midi kit (Qiagen) according to the manufacturer's instructions. Finally, the DNA

30 was taken up in 100 μ l of TE (10 mM Tris/HCl pH 8.0, 1 mM EDTA) and stored at 4°C for further use.

In order to prepare a library of recombinant phagemids (Kay et al., Phage Display of Peptides and Proteins - A

35 Laboratory Manual (1996), Academic Press) which carry the muteins of the bilin-binding protein as a fusion with the truncated coat protein pIII, the phasmid DNA obtained in this way was used for transformation of

Electroporation was carried out as described above with the aid of the Easyjec T Basic system. In a total of 13 mixtures, 40 μ l of the cell suspension of electrocompetent cells were in each case transformed with in each case 2 μ g of the DNA in a volume of 5 μ l. After electroporation, the cell suspension obtained from each mixture was diluted immediately in 2 ml of fresh ice-cold SOC medium and agitated at 37°C and 200 rpm for 60 min.

10

The mixtures were combined (volume = 26 ml) and 74 ml of 2xYT medium and 100 μ l of ampicillin (stock solution 100 mg/ml, final concentration 100 mg/l) were added. The total number of transformants obtained was estimated at $1.1 \cdot 10^{10}$ by plating out 100 μ l of a 1:10⁵ dilution of the obtained suspension on agar plates containing LB/Amp medium. After incubation at 37°C and 160 rpm for 60 min, the culture was infected with 500 μ l of VCS-M13 helper phage ($1.1 \cdot 10^{12}$ pfu/ml, Stratagene) and agitated at 37°C, 160 rpm for a further 60 min. Subsequently, 200 μ l of kanamycin (stock solution 35 mg/ml, final concentration 70 mg/l) were added, the incubator temperature was lowered to 26°C and, after 10 min, anhydrotetracycline (50 μ l of a 50 μ g/ml stock solution in dimethylformamide, final concentration 25 μ g/l) was added to induce gene expression. Finally, for production of the phagemids the culture was incubated at 26°C, 160 rpm for 7 h.

30

The cells were removed by centrifugation of the culture (15 min, 12000 g, 4°C). The supernatant containing the phagemid particles was sterile-filtered (0.45 μ m), mixed with 1/4 volume (25 ml) of 20% w/v PEG 8000, 15% w/v NaCl and incubated at 4°C overnight. After centrifugation (20 min, 18000 g, 4°C), the precipitated phagemid particles were dissolved in a total of 4 ml of cold PBS (4 mM KH₂PO₄, 16 mM Na₂HPO₄, 115 mM NaCl, pH 7.4). The solution was incubated on ice for 30 min and

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volumes. After removing undissolved components by centrifugation (5 min, 18500 g, 4°C), the supernatant was transferred in each case to a new reaction vessel.

- 5 The phagemid particles were again precipitated by mixing with 1/4 volume (in each case 0.25 ml per reaction vessel) of 20% w/v PEG 8000, 15% w/v NaCl and incubating on ice for 60 min. After centrifugation (20 min, 18500 g, 4°C), the supernatant was removed and
10 the precipitated phagemid particles were each dissolved in 0.5 ml of PBS. After incubation on ice for 30 min, centrifugation (5 min, 18500 g, 4°C) was repeated to clarify the solution. The supernatant containing the phagemid particles (between $1 \cdot 10^{12}$ and $5 \cdot 10^{12}$ cfu/ml) was
15 then used for affinity enrichment.

- For affinity enrichment of the recombinant phagemids presenting the muteins of the bilin-binding protein Immuno-Sticks (NUNC) were used. These were coated
20 overnight with 800 μ l of a conjugate (100 μ g/ml) of ribonuclease A (RNaseA) and digoxigenin in PBS.

- The conjugate was prepared by adding 1.46 μ mol (0.96 mg) of digoxigenin-3-O-methylcarbonyl- ϵ -amino-caproic acid N-hydroxysuccinimide ester (DIG-NHS, Boehringer Mannheim) in 25 μ l of DMSO in μ l steps and with constant mixing to 0.73 μ mol (10 mg) of RNaseA (Fluka) in 1 ml of 5% w/v sodium hydrogen carbonate. The mixture was incubated with stirring at room
25 temperature (RT) for 1 h. Excess reagent was then removed from the RNaseA conjugate by means of a PD-10 gel filtration column (Pharmacia) according to the manufacturer's instructions.
30

- 35 Unoccupied binding sites on the Immuno-Stick surface were saturated by incubation with 1.2 ml of 2% w/v BSA in PBST (PBS with 0.1% v/v Tween 20) at RT for 2 h. After three short washes with in each case 1.2 ml of

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250 μ l of phagemid solution and 500 μ l of blocking buffer (2% w/v BSA in PBST) at RT for 1 h.

For removing unbound phagemids the solution was
5 stripped off and the Immuno-Stick was washed eight times with in each case 950 μ l of PBST for 2 min. Finally, adsorbed phagemids were competitively eluted during a 15 minute incubation of the Immuno-Stick with 950 μ l of a 2 mM solution of digoxigenin in PBS (for
10 this purpose, 0.742 mg of digoxigenin (Fluka) were dissolved in 19.2 μ l of DMF and added to 930.8 μ l of PBS).

The phagemids were propagated by heating 950 μ l of
15 solution of the elution fraction obtained (between 10^6 and 10^8 colony-forming units, depending on the selection cycle) briefly to 37°C, mixing the solution with 4 ml of an exponentially growing culture of *E. coli* XL1-Blue ($OD_{550} = 0.5$) and incubated at 37°C,
20 200 rpm for 30 min. The phagemid-infected cells were then sedimented (2 min, 4420 g, 4°C), resuspended in 800 μ l of fresh 2xYT medium and plated out on four agar plates containing LB/Amp medium (140 mm in diameter). After incubation at 32°C for 14 h, the colonies
25 obtained in this way were scraped off the agar plates with the addition of in each case 10 ml of 2xYT/Amp medium, transferred to a sterile Erlenmeyer flask and agitated at 37°C, 200 rpm for 20 min to complete resuspension.

30

For repeated production and affinity enrichment of phagemid particles 50 ml of 2xYT/Amp medium prewarmed to 37°C were inoculated with 0.2 to 1 ml of said suspension so that the cell density was $OD_{550} = 0.08$.

35 This culture was incubated at 37°C, 160 rpm to a cell density of $OD_{550} = 0.5$, infected with 250 μ l of VCS-M13 helper phage ($1.1 \cdot 10^{12}$ pfu/ml, Stratagene), and the procedure was continued as already described above.

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The phagemids obtained from the first affinity concentration were used to carry out a series of eight further enrichment cycles using Immuno-Sticks which had been freshly coated with the digoxigenin-RNaseA conjugate. The phagemids obtained after the last enrichment cycle were again used for infecting *E. coli* XL1-Blue. The mixture of the colonies obtained was scraped off the agar plates using 2xYT/Amp medium and resuspended, as described above. This cell suspension was used to inoculate 50 ml of 2xYT/Amp medium, and the phasmid DNA was isolated using the QIAprep Spin Miniprep kit (QIAGEN) according to the manufacturer's instructions.

In order to be able to produce the muteins of the bilin-binding protein as a fusion protein with the Strep-tag II and the albumin-binding domain, the gene cassette between the two *Bst*XI cleavage sites was subcloned from vector pBBP20 into vector pBBP22. A relevant section of the pBBP22 nucleic acid sequence is represented, together with the encoded amino acid sequence, as SEQ ID NO:9 in the sequence listing. The section starts with the *Xba*I cleavage site and ends with the *Hind*III cleavage site. The vector elements outside this region are identical to vector pASK75.

For this purpose, the DNA isolated from the mixture of the *E. coli* colonies was cut with restriction enzyme *Bst*XI, and the smaller of the two fragments (335 bp) was purified by preparative agarose gel electrophoresis as described above. In the same manner, pBBP22 vector DNA was cut with *Bst*XI and the larger of the two fragments (3545 bp) was isolated.

1.5 Weiss units of T4 DNA ligase (Promega) were added to 50 fmol of each of the two DNA fragments in a total volume of 20 μ l (30 mM Tris/HCl pH 7.8, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP) and the mixture was incubated for

mixture were used to transform 200 μ l of competent cells of *E. coli* strain TG1-F⁻ according to the CaCl₂ method (Sambrook et al., supra), and 2.2 ml of a cell suspension were obtained.

5 The transformants were then screened for production of mureins with binding activity for the digoxigenin group by means of a colony screening assay. For this purpose, a cut-to-fit hydrophilic PVDF membrane (Millipore, type
10 GVWP, pore size 0.22 μ m) marked at one position was placed on an LB/Amp agar plate. 150 μ l of the cell suspension from the transformation mixture were plated out evenly on said membrane, and approx. 500 colonies were obtained. The plate was incubated in an incubator
15 at 37°C for 7.5 h until the colonies were approx. 0.5 mm in diameter.

In the meantime, a hydrophobic membrane (Millipore, Immobilon P, pore size 0.45 μ m) which had likewise been
20 cut to fit was wetted with PBS according to the manufacturer's instructions. Said membrane was then gently agitated in a solution of 10 mg/ml of human serum albumin (HSA, Sigma) in PBS at RT for 4 h. Remaining binding sites on the membrane were saturated
25 by incubation with 3% w/v BSA, 0.5% v/v Tween 20 in PBS at RT for 2 h. The membrane was washed with 20 ml of PBS for two times for 10 min and then gently agitated in 10 ml of LB/Amp medium to which 200 μ g/l of anhydrotetracycline had been added for 10 min. Said
30 membrane was then marked at one position and placed on a culture plate with LB/Amp agar which additionally contained 200 μ g/l of anhydrotetracycline.

The previously obtained hydrophilic membrane on which
35 colonies had grown was then placed onto the hydrophobic membrane such that the two markings coincided. The culturing plate with the two membranes was incubated at 22°C for 15 h. During this phase, the particular

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proteins and immobilized on the lower membrane by means of complex formation between the albumin-binding domain and the HSA.

- 5 Subsequently, the upper membrane containing the colonies was transferred to a fresh LB/Amp agar plate and stored at 4°C. The hydrophobic membrane was removed, washed with 20 ml of PBST for three times
10 10 min and then incubated in 10 ml of a 10 µg/ml solution of a conjugate of BSA with digoxigenin in PBST for 1 h.

- The conjugate of BSA (Sigma) and digoxigenin was prepared by adding a solution of 3.0 µmol (1.98 mg) of
15 DIG-NHS in 25 µl of DMSO in µl steps and with constant mixing to 300 nmol (19.88 mg) of BSA (Sigma) in 1.9 ml of 5% w/v sodium hydrogen carbonate. The mixture was incubated with stirring at RT for 1 h and excess reagent was removed from the BSA conjugate by means of
20 a PD-10 gel filtration column according to the manufacturer's instructions.

- In order to detect bound digoxigenin-BSA conjugate, the membrane was incubated, after washing twice in 20 ml of
25 PBST, with 10 ml of anti-digoxigenin Fab-alkaline phosphatase conjugate (Boehringer Mannheim, diluted 1:1000 in PBST) for 1 h. The membrane was then washed twice with 20 ml PBST and twice with 20 ml of PBST for in each case 5 min and gently agitated in AP buffer
30 (0.1 M Tris/HCl pH 8.8, 0.1 M NaCl, 5 mM MgCl₂) for 10 min. For the chromogenic detection reaction, the membrane was incubated in 10 ml of AP buffer to which 30 µl of 5-bromo-4-chloro-3-indolyl phosphate, p-toluidinium salt (BCIP, Roth, 50 µg/ml in
35 dimethylformamide) and 5 µl of Nitro Blue Tetrazolium (NBT, Sigma, 75 µg/ml in 70% v/v dimethylformamide) had been added, until at the positions of some of the colonies distinct color signals became visible. In this

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protein muteins which had been produced in the form of fusion proteins with Strep-tag and ABD by said colonies was detected.

5 Four colonies from the upper membrane, which caused a distinct color signal, were used for preparing cultures in LB/Amp medium of 4 ml in volume. Their plasmid DNA was isolated with the aid of the JETquick Plasmid Miniprep Spin kit (Genomed) according to the
10 manufacturer's instructions, and the gene section coding for the mutein was subjected to sequence analysis. Sequence analysis was carried out with the aid of the T7 sequencing kit (Pharmacia) according to the manufacturer's instructions by using
15 oligodeoxynucleotides SEQ ID NO:10 and SEQ ID NO:11. It was found in the process that all four plasmids studied carried the same nucleotide sequence. The corresponding gene product was denoted by DigA (SEQ ID NO:12). The DigA nucleotide sequence was translated into the amino
20 acid sequence and is represented in the sequence listing.

Example 2: Partial random mutagenesis of the DigA
mutein and selection of muteins with improved binding
25 affinity for digoxigenin

In order to improve the affinity between the DigA mutein and digoxigenin, which was determined as 295 ± 36 nM according to Example 3, the 6 amino acid
30 positions 28, 31 and 34-37 in DigA were selected for a more substantial partial random mutagenesis.

For mutating said positions the PCR was carried out using a degenerated oligodeoxynucleotide primer. The
35 amplification reaction was carried out in a total volume of 100 μ l, with 2 ng of the vector pBBP22 plasmid DNA coding for DigA (SEQ ID NO:12) being used as template. The reaction mixture contained 50 pmol of

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the other components according to the method described in Example 1. The PCR was carried out in 20 temperature cycles of 1 min at 94°C, 1 min at 65°C, and 1.5 min at 72°C, followed by a final incubation at 60°C for 5 min.

5 The DNA fragment obtained was isolated by preparative agarose gel electrophoresis and then cut with BstXI according to the manufacturer's instructions. The resulting DNA fragment of 335 bp in length was again purified by preparative agarose gel electrophoresis.

10

The pBBP24 vector DNA was cut with BstXI accordingly and the 4028 bp fragment obtained was isolated. A relevant section of the pBBP24 nucleic acid sequence is represented, together with the encoded amino acid sequence, as SEQ ID NO:14 in the sequence listing. The section starts with the XbaI cleavage site and ends with the HindIII cleavage site. The vector elements outside this region are identical to vector pASK75. PBBP24 is virtually identical with pBBP20, wherein the BBP gene has been inactivated by means of appropriately introduced stop codons.

20

1.3 µg of the cleaved DNA fragment from the PCR and 16.0 µg of the pBBP24 fragment were incubated for ligation in the presence of 120 Weiss units of T4 DNA ligase (New England Biolabs) in a total volume of 600 µl (50 mM Tris/HCl pH 7.8, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 50 µg/ml BSA) at 16°C for 18 h. The DNA was then precipitated by adding 10 µg of yeast tRNA (Boehringer Mannheim), 25 µl of 5 M ammonium acetate and 100 µl of ethanol to in each case 24 µl of the ligation mixture. Incubation at -20°C for two weeks was followed by centrifugation (20 min, 16000 g, 4°C). The precipitate was washed in each case with 150 µl of ethanol (70% v/v, -20°C) and dried under vacuum. Finally, the DNA was taken up in 80 µl of TE/10.

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E. coli XL1-Blue cells were transformed with the

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procedure described in Example 1, with in each case 40 μ l of cell suspension of electrocompetent cells being mixed with 5 μ l of the DNA solution in 16 mixtures. After electroporation, the cells were
5 immediately diluted in 2 ml of fresh ice-cold SOC medium and agitated at 37°C and 200 rpm for 60 min.

168 ml of 2xYT medium and 200 μ l of ampicillin (stock solution 100 mg/ml, final concentration 100 mg/l) were
10 added to the combined suspensions. The total number of transformants obtained was estimated at $1.48 \cdot 10^9$ by plating out 100 μ l of a $1:10^4$ dilution of the obtained cell suspension on agar plates. After incubation at 37°C and 160 rpm for 60 min, the transformants were
15 infected with 4 ml of VCS-M13 helper phage ($6.3 \cdot 10^{11}$ pfu/ml, Stratagene) and agitated at 37°C and 160 rpm for a further 30 min. Subsequently, 400 μ l of kanamycin (stock solution 35 mg/ml, final concentration 70 mg/l) were added, the incubator temperature was lowered to
20 26°C and, after 10 min, anhydrotetracycline (100 μ l of a 50 μ g/ml stock solution in dimethylformamide, final concentration 25 μ g/l) was added to induce gene expression. Finally, the phagemids were produced by incubating the culture at 26°C and 160 rpm for 7 h. The
25 cells were removed and the phagemids purified by precipitation as described in Example 1.

Streptavidin-coated paramagnetic particles (Dynabeads M-280 Streptavidin, Dynal) were used together with a
30 double conjugate of BSA with digoxigenin and biotin for affinity enrichment from the library of phagemids which presented the partially mutated DigA mutein.

A double conjugate of BSA with digoxigenin and biotin
35 was prepared by adding 1.5 μ mol (0.99 mg) of DIG-NHS in 12.5 μ l of DMSO and 1.5 μ mol (0.68 mg) of D-biotinoyl- ϵ -aminocaproic acid N-hydroxysuccinimide ester (Boehringer Mannheim) in 12.5 μ l of DMSO in μ l

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of BSA in 1.9 ml of 5% w/v sodium hydrogen carbonate. The mixture was incubated with stirring at RT for 1 h. Excess reagent was removed from the double conjugate via a PD-10 gel filtration column according to the
5 manufacturer's instructions.

In order to enrich Digoxigenin-binding phagemids, 40 μ l of a 0.5 μ M solution of the double conjugate (33.5 μ g/ml) in PBS were mixed with 260 μ l of a
10 solution of the freshly prepared phagemids (between $5 \cdot 10^{11}$ and $5 \cdot 10^{12}$ cfu/ml) and incubated at RT for 1 h so that the complex formation between the digoxigenin group and the muteins presented by the phagemids was able to occur. This was followed by adding 100 μ l of a
15 solution of 8% w/v BSA, 0.4% v/v Tween 20 in PBS.

Parallel thereto, 100 μ l of the commercially available suspension of paramagnetic particles were washed with three times 100 μ l of PBS. Here, the particles were
20 kept suspended for 1 min by rotating the 1.5 ml Eppendorf vessel and then collected at the wall of the Eppendorf vessel with the aid of a magnet, and the supernatant was stripped off. In order to saturate unspecific binding sites, the paramagnetic particles
25 were incubated with 100 μ l of 2% w/v BSA in PBST at RT for 1 h. After removing the supernatant, the mixture of double conjugate and phagemids was added to the paramagnetic particles, and the particles were resuspended and incubated at RT for 10 min. Finally,
30 free biotin-binding sites of Streptavidin were saturated by adding 10 μ l of a 4 μ M D-desthiobiotin (Sigma) solution in PBS to the mixture and incubating said mixture at RT for 5 min. This procedure also
prevented the Strep-tag II as part of the fusion
35 protein of the muteins and the phage coat protein pIII fragment from being able to form a complex with Streptavidin.

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Unbound phagemids were removed by washing the paramagnetic particles eight times with 1 ml of fresh PBST with the addition of 1 mM D-desthiobiotin, the particles were collected with the aid of the magnet and the supernatant was stripped off. The bound phagemids were eluted by incubating the resuspended particles in 950 μ l of 0.1 M glycine/HCl pH 2.2 for 15 minutes. After collecting the particles on the magnet, the supernatant was again stripped off and this was immediately followed by neutralizing the pH of said solution by addition of 140 μ l of 0.5 M Tris.

The phagemids were propagated by mixing the elution fraction obtained, according to the procedure in Example 1, with 4 ml of an exponentially growing culture of *E. coli* XL1-Blue ($OD_{550} = 0.5$) and incubating at 37°C, 200 rpm for 30 min. The phagemid-infected cells were then sedimented (2 min, 4420 g, 4°C), resuspended in 800 μ l of fresh 2xYT medium and plated out on four agar plates containing LB/Amp medium (140 mm in diameter). After incubation at 32°C for 14 h, the colonies obtained in this way were scraped off the agar plates with the addition of in each case 10 ml of 2xYT/Amp medium, transferred to a sterile Erlenmeyer flask and agitated at 37°C, 200 rpm for 20 min to complete resuspension.

For repeated production and affinity enrichment of phagemid particles 50 ml of 2xYT/Amp medium prewarmed to 37°C was inoculated with 0.2 to 1 ml of said suspension so that the cell density was $OD_{550} = 0.08$. This culture was incubated at 37°C, 160 rpm to a cell density of $OD_{550} = 0.5$ and infected with 300 μ l of VCS-M13 helper phage ($6.3 \cdot 10^{11}$ pfu/ml, Stratagene). The affinity selection was then repeated using the paramagnetic particles and the digoxigenin/biotin double conjugate under the abovementioned conditions. A total of 4 selection cycles were carried out in this

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The phagemids obtained after the last concentration cycle were again used for infecting *E. coli* XL1-Blue. The mixture of the obtained colonies which had been
5 scraped off the agar plates using 2xYT/Amp medium and had been resuspended, as described above, was used to inoculate 50 ml of 2xYT/Amp medium, and phasmid DNA was isolated using the QIAprep spin miniprep kit (QIAGEN) according to the manufacturer's instructions.

10 Subsequently, the gene cassette between the two *Bst*XI cleavage sites was subcloned, as in Example 1, from vector pBBP24 into vector pBBP22, and competent cells of *E. coli* strain TG1-F⁻ were transformed according to
15 the CaCl₂ method. Finally, the transformants were, again according to Example 1, screened for production of muteins with binding activity for the digoxigenin group by means of the colony screening assay.

20 Seven of the colonies showing a strong signal intensity in the colony screening assay were cultured. Their plasmid DNA was isolated by means of the plasmid miniprep spin kit (Genomed) according to the manufacturer's instructions, and the gene section
25 coding for the mutein was subjected to sequence analysis as in Example 1. It was found in the process that all plasmids studied had different sequences. After translating the nucleotide sequences into amino acid sequences, six of the seven variants studied had
30 an amber stop codon at amino acid position 28. However, this stop codon was at least partially suppressed when choosing suitable amber-suppressor strains such as, for example, *E. coli* XL1-Blue or TG1-F⁻ and instead translated as glutamine. Thus a full-length functional
35 protein was produced both during affinity enrichment and in the colony screening assay.

As the only mutein without an amber stop codon among

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particularly well suited for bacterial production. Consequently, this mutein, also denoted by DigA16, was characterized in more detail with regard to its ability to bind to the digoxigenin group.

5

Example 3: Production of the DigA and DigA16 muteins and determination of their affinity for digoxigenin and derivatives thereof by fluorescence titration

10 For preparative production of the bilin-binding protein muteins obtained from the previous Examples the coding gene section between the two *Bst*XI cleavage sites was subcloned from the type pBBP22 vector into the expression plasmid pBBP21. The plasmid thus obtained
15 coded for a fusion protein of the OmpA signal sequence, followed by the mutein and the Strep-tag II affinity tag.

A relevant section of the pBBP21 nucleic acid sequence
20 is represented, together with the encoded amino acid sequence, as SEQ ID NO:16 in the sequence listing. The section starts with the *Xba*I cleavage site and ends with a hexanucleotide which was obtained by ligating a blunt strand end with a filled-up *Hind*III strand end,
25 with the loss of the original *Hind*III cleavage site. The vector elements outside this region are identical to vector pASK75.

For subcloning, the plasmid DNA coding for the relevant
30 mutein was cut with restriction enzyme *Bst*XI, and the smaller of the two fragments (335 bp) was purified by preparative agarose gel electrophoresis as described in Example 1. In the same manner, pBBP21 vector DNA was cut with *Bst*XI, and the larger of the two fragments
35 (4132 bp) was isolated.

1.5 Weiss units of T4 DNA ligase (Promega) were added to 50 fmol of each of the two DNA fragments in a total

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10 mM DTT, 1 mM ATP) and the mixture was incubated for ligation at 16°C for 16 h. 5 µl of the ligation mixture were then used to transform *E. coli* JM83 (Yanisch-Perron et al., Gene 33 (1985), 103-119) according to the CaCl₂ method, wherein 2.2 ml of a cell suspension were obtained. 100 µl of this suspension were plated out on an agar plate containing LB/Amp medium and incubated at 37°C for 14 h.

10 For protein production, one of the obtained single colonies was selected, a 50 ml preculture (LB/Amp medium) was inoculated with this colony and incubated at 30°C and 200 rpm overnight. 40 ml of the preculture were then transferred by inoculating 2 l of LB/Amp medium in a 5 l Erlenmeyer flask, followed by incubating the culture at 22°C and 200 rpm. At a cell density of OD₅₅₀ = 0.5, gene expression was induced by adding 200 µg/l anhydrotetracycline (200 µl of a 2 mg/ml stock solution in DMF), followed by agitating at 22°C, 200 rpm for a further 3 h.

The cells were removed by centrifugation (15 min, 4420 g, 4°C) and, after removing the supernatant, resuspended in 20 ml of periplasm lysis buffer (100 mM Tris/HCl pH 8.0, 500 mM sucrose, 1 mM EDTA) with cooling on ice. After incubation on ice for 30 min, the spheroplasts were removed in two successive centrifugation steps (15 min, 4420 g, 4°C and 15 min, 30 000 g, 4°C). The periplasmic protein extract obtained in this way was dialyzed against SA buffer (100 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA), sterile-filtered and used for chromatographic purification.

35 Purification was carried out by means of the Strep-tag II affinity tag (Schmidt and Skerra, Protein Eng. 6 (1993), 109-122) fused to the C-terminus of the muteins. In the present case, Streptavidinmutedin "1"

982), which was coupled to activated Sepharose (with 5 mg/ml immobilized Streptavidin with respect to the bed volume of the matrix).

5 A chromatography column packed with 2 ml of said material was equilibrated at 4°C and a flow rate of 20 ml/h with 10 ml of SA buffer. The chromatography was monitored by measuring absorption of the eluate at 280 nm in a flow-through photometer. Application of the
10 periplasmic protein extract was followed by washing with SA buffer until the base line was reached. Bound mutein was then eluted with 10 ml of a solution of 2.5 mM D-desthiobiotin (Sigma) in SA buffer. The fractions containing the purified mutein were checked
15 by means of SDS polyacrylamide gel electrophoresis (Fling and Gregerson, Anal. Biochem. 155 (1986), 83-88) and combined. The protein yields were between 200 µg and 800 µg per 2 l culture.

20 The ligand binding properties of muteins DigA, DigA16 and also of the recombinant bilin-binding protein (SEQ ID NO:16) were determined by means of the method of fluorescence titration. In this case, the decrease in intrinsic tyrosine and/or tryptophan fluorescence of
25 the protein forming a complex with the ligand was measured. The measurements were carried out in a fluorimeter, type LS 50 B (Perkin Elmer) at an excitation wavelength of 295 nm (slit width 4 nm) and an emission wavelength of 345 nm (slit width 6 nm). The
30 ligands used were digoxigenin (Fluka), digoxin (Fluka), digitoxigenin (Fluka), digitoxin (Fluka), testosterone (Sigma), ouabain (Fluka), and 4-aminofluorescein (Fluka). The ligands showed no significant intrinsic fluorescence or absorption at the stated wavelength.

35

The buffer system used was PBS with the addition of 1 mM EDTA. The solution of the relevant purified mutein was dialyzed four times against this buffer and

- 40 -

solutions used were sterile-filtered (Filtropur S 0.45 μm , Sarstedt). The concentration was determined by means of absorption at 280 nm using calculated extinction coefficients of 53580 $\text{M}^{-1} \text{cm}^{-1}$ for DigA and DigA16 (Wisconsin Software Package, Genetics Computer Group). For Bbp, the calculated extinction coefficient of 54150 $\text{M}^{-1} \text{cm}^{-1}$, corrected in the presence of guanidinium chloride according to Gill and von Hippel (Anal. Biochem. 182 (1989), 319-326) was used.

For the measurement, 2 ml of the mutein solution were introduced into a quartz cuvette equipped with a magnetic stirrer bar and thermally equilibrated at 25°C in the sample holder of the photometer. Then a total of 40 μl of a 100 μM to 500 μM solution of the ligand in the same buffer were pipetted in steps of from 1 μl to 4 μl . The dilution of the introduced protein solution by altogether no more than 2%, which took place in the process, was not taken into account in the subsequent evaluation of the data. After each titration step, the equilibrium was allowed to reach by incubating with stirring for 1 min, and the fluorescence signal was measured as average over 10 s. After subtracting the fluorescence value of the buffer, the signals were normalized to an initial value of 100%.

The thus obtained data of a titration series were fitted by nonlinear regression using the computer program Kaleidagraph (Abelbeck Software) according to the following equation

$$F = ([P]_t - [L]_t - K_d) \frac{f_p}{2} + ([P]_t + [L]_t + K_d) \frac{f_{pL}}{2} + (f_p - f_{pL}) \sqrt{\frac{([P]_t + [L]_t + K_d)^2}{4} - [P]_t [L]_t}$$

Here, F means the normalized fluorescence intensity and $[L]_t$ the total ligand concentration in the particular titration step. $[P]_t$ as mutein concentration, f_{pL} as

- 41 -

and K_d as the thermodynamic dissociation constant of said complex were fitted as free parameters to the normalized data.

- 5 Figure 1 represents graphically the results of the fluorescence titrations of the DigA16 mutein with the ligands digoxigenin, digitoxigenin and ouabain. It turns out that digitoxigenin is bound even tighter than digoxigenin, while no binding is observed for ouabain.

10

The values resulting from the fluorescence titrations for the dissociation constants of the complexes of the bilin-binding protein muteins and the various ligands are summarized in the following table:

15

<u>Bbp variant</u>	<u>Ligand</u>	<u>K_d [nM]</u>
Bbp:	digoxigenin	-*
DigA:	digoxigenin	295 ± 37
	digoxin	200 ± 34
20 DigA16:	digoxigenin	30.2 ± 3.6
	digoxin	31.1 ± 3.2
	digitoxigenin	2.8 ± 2.7
	digitoxin	2.7 ± 2.0
	ouabain	-*
25	testosterone	-*
	4-aminofluorescein	-*

no detectable binding activity

- 30 Example 4: Preparation of fusion proteins of the DigA16 mutein and bacterial alkaline phosphatase and use for detecting digoxigenin groups in an ELISA and in a Western blot

- 35 In order to produce two different fusion proteins of the DigA16 mutein and bacterial alkaline phosphatase (PhoA) with different arrangement of the partners within the polypeptide chain, the two expression

the molecular-biological methods familiar to the skilled worker.

pBBP27 codes for a fusion protein of PhoA including the
5 signal sequence thereof, a short peptide linker having
the amino acid sequence Pro-Pro-Ser-Ala, the sequence
corresponding to the mature DigA16 mutein and the
Strep-tag-II. A relevant section of the pBBP27 nucleic
acid sequence is represented, together with the encoded
10 amino acid sequence, as SEQ ID NO:17 in the sequence
listing. The section begins with the *Xba*I cleavage site
and ends with the *Hind*III cleavage site. The vector
elements outside this region are identical to vector
pBBP21.

15 pBBP29 codes for a fusion protein of DigA16 with
preceding OmpA signal sequence, followed by the peptide
sequence for Strep-tag II, a sequence of 5 glycine
residues and the mature PhoA sequence without the N-
20 terminal amino acid arginine. A relevant section of the
pBBP29 nucleic acid sequence is represented, together
with the encoded amino acid sequence, as SEQ ID NO:18
in the sequence listing. The section begins with the
*Xba*I cleavage site and ends with the *Hind*III cleavage
25 site. The vector elements outside this region are
identical to vector pBBP21.

Both plasmids additionally code for the bacterial
protein disulfide isomerase DsbC on a separate cistron
30 located in 3' direction. The plasmids are shown
diagrammatically in Figure 2.

The fusion proteins encoded by plasmids pBBP27 and
pBBP29 were produced analogously to the method for
35 preparing the simple muteins, described in Example 3.
In order to avoid complexing the metal ions from the
active center of PhoA, lysis of the bacterial periplasm
was carried out using EDTA-free lysis buffer.

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buffer as an agent destabilizing the outer cell membrane. All other buffers used for purification were likewise EDTA-free.

5 The fusion proteins purified by affinity chromatography by means of the Strep-tag II were dialyzed against PBS buffer overnight. The fusion protein yields were between 100 and 200 μg per 2 l of culture medium. The purity of the fusion proteins obtained was checked by
10 SDS polyacrylamide gel electrophoresis, according to Example 3, and determined to be 90-95%. Subsequently, the fusion proteins were used for directly detecting conjugates of the digoxigenin group with various proteins both in a sandwich ELISA and in a Western
15 blot.

While the conjugates used of digoxigenin with RNaseA and BSA were prepared according to Example 1, a conjugate of digoxigenin with ovalbumin (Sigma) was
20 prepared by adding 1.5 μmol (0.99 mg) DIG-NHS in 25 μl of DMSO in μl steps and with constant mixing to 300 nmol (13.5 mg) of ovalbumin in 1.9 ml of 5% sodium hydrogen carbonate. The mixture was incubated with stirring at RT for 1 h. Excess reagent was removed from
25 the ovalbumin conjugate via a PD-10 gel filtration column according to the manufacturer's instructions.

For detecting digoxigenin groups in a sandwich ELISA, the wells in in each case two rows of a microtiter
30 plate (ELISA strips, 2x8 well with high binding capacity, F-type, Greiner) were filled in each case with 100 μl of a 100 $\mu\text{g}/\text{ml}$ solution of the BSA-digoxigenin conjugate or the ovalbumin-digoxigenin conjugate in PBS and incubated at RT overnight. As a
35 control, the wells of a fifth vertical row of the microtiter plate were filled with 100 μl of a 100 $\mu\text{g}/\text{ml}$ solution of nonconjugated BSA (Sigma) in PBS and likewise incubated at RT overnight. After removing the

- 44 -

200 μ l of a solution of 2% w/v BSA in PBST for 2 h. After washing three times with PBST, 50 μ l of a 1 μ M solution of the purified fusion protein were in each case introduced into the first well of a row, and the
5 Tween concentration was adjusted to 0.1% v/v by adding 1 μ l of a solution of 5% v/v Tween. The subsequent wells in each row were initially charged with 50 μ l of PBST. Then, 50 μ l of the purified fusion protein were pipetted in each case into the second well, mixed and,
10 starting therefrom, 1:2 dilutions were prepared stepwise in the other wells of the vertical row. After incubation at RT for 1 h, the wells were washed twice with PBST and twice with PBS. The fusion proteins bound to the digoxigenin groups were finally detected by
15 means of alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate. For this purpose, 100 μ l of a solution of 0.5 mg/ml p-nitrophenyl phosphate (Amresco) in AP buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris/HCl pH 8.8) were introduced into the wells and product
20 formation was monitored by measuring absorption at 405 nm in a SpectraMax 250 photometer (Molecular Devices).

Figure 3 shows the result of this measurement.
25 According to this, the digoxigenin group is recognized both as conjugate with BSA and as conjugate with ovalbumin, leading to the conclusion that binding by the DigA16 mutein is context-independent. Furthermore, both fusion proteins are active both with regard to the
30 binding function for the digoxigenin group and enzymatically and produce, despite their different structure, almost identical signals.

In order to use the fusion proteins encoded by vectors
35 pBBP27 and pBBP29 in a Western blot, 5 μ l of a protein mixture in PBS, whose concentration of digoxigenin-BSA conjugate, digoxigenin-ovalbumin conjugate and digoxigenin-RNaseA conjugate was simultaneously in each

- 45 -

PBS, whose concentration of underivatized BSA, ovalbumin and RNaseA likewise was simultaneously in each case 100 $\mu\text{g/ml}$, were first separated by SDS polyacrylamide gel electrophoresis. The protein mixture was then transferred to nitrocellulose by electrotransfer (Blake et al., Anal. Biochem. 136 (1984), 175-179). The membrane was then washed in 10 ml of PBST for three times 5 min and incubated with 10 ml of a 0.5 μM solution of in each case one of the two fusion proteins for 1 h. The membrane was then washed in 10 ml PBST for two times 5 min and in 10 ml of PBS for two times 5 min and finally gently agitated in 10 ml of AP buffer for 10 min. For the chromogenic detection reaction, the membrane was incubated in 10 ml of AP buffer to which 30 μl BCIP (50 $\mu\text{g/ml}$ in dimethylformamide) and 5 μl NBT (75 $\mu\text{g/ml}$ in 70% v/v dimethylformamide) had been added, and bound fusion protein was detected in this way.

Figure 4 shows the result of this detection method. It turns out again that binding of the digoxigenin group by the two fusion proteins is independent of the carrier protein and that both fusion proteins achieve comparable signal intensities. The same carrier proteins cause no signal whatsoever if they are not conjugated with the digoxigenin group.

SEQUENCE LISTINGS

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5 <120> Muteins of bilin-binding protein

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 50 55 60

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 His Gly Lys Glu Tyr Phe Ile Glu Gly Thr Ala Tyr Pro Val Gly
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 230

40

<210> 10

<211> 17 bases

<212> DNA

45 <213> artificial sequence

<220>

<223> sequencing primer

<400> 10

c n

<210> 11
 <211> 17 bases
 <212> DNA
 5 <213> artificial sequence

 <220>
 <223> sequencing primer

 10 <400> 11

 GACTACTGGG GAGCCGA 17

 15 <210> 12
 <211> 522 bases
 <212> DNA
 <213> coding sequence of mutein DigA

 20 <220>
 <221> CDS
 <222> (1)...(522)
 <223> mutein DigA without fusions parts

 25 <400> 12

 GAC GTG TAC CAC GAC GGT GCC TGT CCC GAA GTC AAG CCA GTC GAC 45
 Asp Val Tyr His Asp Gly Ala Cys Pro Glu Val Lys Pro Val Asp
 1 5 10 15
 30 AAC TTC GAC TGG TCC CAG TAC CAT GGT AAA TGG TGG GAA GTC GCC 90
 Asn Phe Asp Trp Ser Gln Tyr His Gly Lys Trp Trp Glu Val Ala
 20 25 30
 35 AAA TAC CCC CAT CAC GAG CGG AAG TAC GGA AAG TGC GGA TGG GCT 135
 Lys Tyr Pro His His Glu Arg Lys Tyr Gly Lys Cys Gly Trp Ala
 35 40 45
 40 GAG TAC ACT CCT GAA GGC AAG AGT GTC AAA GTT TCG CGC TAC TCT 180
 Glu Tyr Thr Pro Glu Gly Lys Ser Val Lys Val Ser Arg Tyr Ser
 50 55 60

10

Val Ile His Gly Lys Glu Tyr Phe Ser Glu Gly Thr Ala Tyr Pro
 65 70 75
 GTT GGT GAC TCC AAG ATT GGA AAG ATC TAC CAC AGC TAC ACT ATT 270
 5 Val Gly Asp Ser Lys Ile Gly Lys Ile Tyr His Ser Tyr Thr Ile
 80 85 90
 GGA GGT GTG ACC CAG GAG GGT GTA TTC AAC GTA CTC TCC ACT GAC 315
 10 Gly Gly Val Thr Gln Glu Gly Val Phe Asn Val Leu Ser Thr Asp
 95 100 105
 AAC AAG AAC TAC ATC ATC GGA TAC TTT TGC TCG TAC GAC GAG GAC 360
 Asn Lys Asn Tyr Ile Ile Gly Tyr Phe Cys Ser Tyr Asp Glu Asp
 110 115 120
 15 AAG AAG GGA CAC ATG GAC TTG GTC TGG GTG CTC TCC AGA AGC ATG 405
 Lys Lys Gly His Met Asp Leu Val Trp Val Leu Ser Arg Ser Met
 125 130 135
 20 GTC CTT ACT GGT GAA GCC AAG ACC GCT GTC GAG AAC TAC CTT ATC 450
 Val Leu Thr Gly Glu Ala Lys Thr Ala Val Glu Asn Tyr Leu Ile
 140 145 150
 GGC TCC CCA GTA GTC GAC TCC CAG AAA CTG GTA TAC AGT GAC TTC 495
 25 Gly Ser Pro Val Val Asp Ser Gln Lys Leu Val Tyr Ser Asp Phe
 155 160 165
 TCT GAA GCC GCC TGC AAG GTC AAC AAT 522
 30 Ser Glu Ala Ala Cys Lys Val Asn Asn
 170
 <210> 13
 <211> 76 bases
 35 <212> DNA
 <213> artificial sequence
 <220>
 <223> primer
 40
 <400> 13

CTGGTCCCAG TACCATGGTA AATGGTGGNN KGTCGCCNNK TACCCCNKN 50
 NKNNKNNKAA GTACGGAAAG TGC GGA 76

45

- <211> 1219 bases pairs
 <212> DNA
 <213> fragment of phasmids pBBP24
- 5 <220>
 <221> sig_peptide
 <222> (22)...(84)
- <220>
- 10 <221> mat_peptide
 <222> (85)...(1209)
 <223> fusion protein of bilin-binding protein, Strep-Tag II and fragment of phage coat protein pIII, with interrupted reading frame
- 15 <220>
 <221> CDS
 <222> (85)...(606)
 <223> mature bilin-binding protein with interrupted reading frame
- 20 <220>
 <221> CDS
 <222> (607)...(636)
 <223> Strep-Tag II affinity tag
- 25 <220>
 <221> CDS
 <222> (637)...(639)
 <223> amber stop codon
- 30 <220>
 <221> CDS
 <222> (640)...(1209)
 <223> amino acids 217-406 of coat protein-pIII
- 35 <400> 14

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	Ala	Val	Ala	Leu	Ala	Gly	Phe	Ala	Thr	Val	Ala	Gln	Ala	Asp	Val	
			-10						-5				-1	1		
10	TAC	CAC	GAC	GGT	GCC	TGT	CCC	GAA	GTC	AAG	CCA	GTC	GAC	AAC	TTC	135
	Tyr	His	Asp	Gly	Ala	Cys	Pro	Glu	Val	Lys	Pro	Val	Asp	Asn	Phe	
			5					10					15			
15	GAC	TGG	TCC	CAG	TAC	CAT	GGT	AAA	TGG	TGG	GAA	GTC	GCC	AAA	TAC	180
	Asp	Trp	Ser	Gln	Tyr	His	Gly	Lys	Trp	Trp	Glu	Val	Ala	Lys	Tyr	
			20					25					30			
20	CCC	AAC	TCA	GTT	GAG	AAG	TAC	GGA	AAT	TAA	TGA	TGG	GCT	GAG	TAC	225
	Pro	Asn	Ser	Val	Glu	Lys	Tyr	Gly	Asn				Trp	Ala	Glu	Tyr
			35					40					45			
25	ACT	CCT	GAA	GGC	AAG	AGT	GTC	AAA	GTT	TCG	AAC	TAC	CAC	GTA	ATC	270
	Thr	Pro	Glu	Gly	Lys	Ser	Val	Lys	Val	Ser	Asn	Tyr	His	Val	Ile	
			50					55					60			
30	CAC	GGC	AAG	GAA	TAC	TTT	ATT	GAA	GGA	ACT	GCC	TAC	CCA	GTT	GGT	315
	His	Gly	Lys	Glu	Tyr	Phe	Ile	Glu	Gly	Thr	Ala	Tyr	Pro	Val	Gly	
			65					70					75			
35	GAC	TCC	AAG	ATT	GGA	AAG	ATC	TAC	CAC	AGC	CTG	ACT	TAC	GGA	GGT	360
	Asp	Ser	Lys	Ile	Gly	Lys	Ile	Tyr	His	Ser	Leu	Thr	Tyr	Gly	Gly	
			80					85					90			
40	GTC	ACC	AAG	GAG	AAC	GTA	TTC	AAC	GTA	CTC	TCC	ACT	GAC	AAC	AAG	405
	Val	Thr	Lys	Glu	Asn	Val	Phe	Asn	Val	Leu	Ser	Thr	Asp	Asn	Lys	
			95					100					105			
45	AAC	TAC	ATC	ATC	GGA	TAC	TAC	TGC	AAA	TAC	GAC	GAG	GAC	AAG	AAG	450
	Asn	Tyr	Ile	Ile	Gly	Tyr	Tyr	Cys	Lys	Tyr	Asp	Glu	Asp	Lys	Lys	
			110					115					120			
50	GGA	CAC	CAA	GAC	TTC	GTC	TGG	GTG	CTC	TCC	AGA	AGC	ATG	GTC	CTT	495
	Gly	His	Gln	Asp	Phe	Val	Trp	Val	Leu	Ser	Arg	Ser	Met	Val	Leu	
			125					130					135			
55	ACT	GGT	GAA	GCC	AAG	ACC	GCT	GTC	GAG	AAC	TAC	CTT	ATC	GGC	TCC	540
	Thr	Gly	Glu	Ala	Lys	Thr	Ala	Val	Glu	Asn	Tyr	Leu	Ile	Gly	Ser	
			140					145					150			
60	CCA	GTA	GTC	GAC	TCC	CAG	AAA	CTG	GTA	TAC	AGT	GAC	TTC	TCT	GAA	585
	Pro	Val	Val	Asp	Ser	Gln	Lys	Leu	Val	Tyr	Ser	Asp	Phe	Ser	Glu	
			155					160					165			
65	GCC	GCC	TGC	AAG	GTC	AAC	AAT	AGC	AAC	TGG	TCT	CAC	CCG	CAG	TTC	630
	Ala	Ala	Cys	Lys	Val	Asn	Asn	Ser	Asn	Trp	Ser	His	Pro	Gln	Phe	
			170					175					180			
70	GAA	AAA	TAG	GCT	GGC	GGC	GGC	TCT	GGT	GGT	GGT	TCT	GGC	GGC	GGC	675

	185	190	195	
5	TCT GAG GGT GGT GGC TCT GAG GGT GGC GGT TCT GAG GGT GGC GGC 720 Ser Glu Gly Gly Gly Ser Glu Gly Gly Gly Ser Glu Gly Gly Gly	200	205	210
10	TCT GAG GGA GGC GGT TCC GGT GGT GGC TCT GGT TCC GGT GAT TTT 765 Ser Glu Gly Gly Gly Ser Gly Gly Gly Ser Gly Ser Gly Asp Phe	215	220	225
15	GAT TAT GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA 810 Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu	230	235	240
20	AAT GCC GAT GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT 855 Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu	245	250	255
25	GAT TCT GTC GCT ACT GAT TAC GGT GCT GCT ATC GAT GGT TTC ATT 900 Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile	260	265	270
30	GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT GGT GCT ACT GGT GAT 945 Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly Ala Thr Gly Asp	275	280	285
35	TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT GAC GGT GAT 990 Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp	290	295	300
40	AAT TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT TCC CTC 1035 Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu	305	310	315
45	CCT CAA TCG GTT GAA TGT CGC CCT TTT GTC TTT GGC GCT GGT AAA 1080 Pro Gln Ser Val Glu Cys Arg Pro Phe Val Phe Gly Ala Gly Lys	320	325	330
50	CCA TAT GAA TTT TCT ATT GAT TGT GAC AAA ATA AAC TTA TTC CGT 1125 Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg	335	340	345
	GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA 1170 Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val	350	355	360
	TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT 1209 Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser	365	370	375
	TAATAAGCTT			1219

<210> 15

<211> 522 bases pairs

<212> DNA

<213> coding sequence of mutein DigA16

5

<220>

<221> CDS

<222> (1)...(522)

<223> mutein DigA16 without fusion parts

10

<400> 15

	GAC GTG TAC CAC GAC GGT GCC TGT CCC GAA GTC AAG CCA GTC GAC	45
	Asp Val Tyr His Asp Gly Ala Cys Pro Glu Val Lys Pro Val Asp	
15	1 5 10 15	
	AAC TTC GAC TGG TCC CAG TAC CAT GGT AAA TGG TGG CAG GTC GCC	90
	Asn Phe Asp Trp Ser Gln Tyr His Gly Lys Trp Trp Gln Val Ala	
	20 25 30	
20	GCG TAC CCC GAT CAT ATT ACG AAG TAC GGA AAG TGC GGA TGG GCT	135
	Ala Tyr Pro Asp His Ile Thr Lys Tyr Gly Lys Cys Gly Trp Ala	
	35 40 45	
25	GAG TAC ACT CCT GAA GGC AAG AGT GTC AAA GTT TCG CGC TAC TCT	180
	Glu Tyr Thr Pro Glu Gly Lys Ser Val Lys Val Ser Arg Tyr Ser	
	50 55 60	
30	GTA ATC CAC GGC AAG GAA TAC TTT TCC GAA GGT ACC GCC TAC CCA	225
	Val Ile His Gly Lys Glu Tyr Phe Ser Glu Gly Thr Ala Tyr Pro	
	65 70 75	
	GTT GGT GAC TCC AAG ATT GGA AAG ATC TAC CAC AGC TAC ACT ATT	270
	Val Gly Asp Ser Lys Ile Gly Lys Ile Tyr His Ser Tyr Thr Ile	
35	80 85 90	
	GGA GGT GTG ACC CAG GAG GGT GTA TTC AAC GTA CTC TCC ACT GAC	315
	Gly Gly Val Thr Gln Glu Gly Val Phe Asn Val Leu Ser Thr Asp	
	95 100 105	
40	AAC AAG AAC TAC ATC ATC GGA TAC TTT TGC TCG TAC GAC GAG GAC	360
	Asn Lys Asn Tyr Ile Ile Gly Tyr Phe Cys Ser Tyr Asp Glu Asp	
	110 115 120	
45	AAG AAG GGA CAC ATG GAC TTG GTC TGG GTG CTC TCC AGA AGC ATG	405
	Lys Lys Gly His Met Asp Leu Val Trp Val Leu Ser Arg Ser Met	
	125 130 135	
	GTC CTT ACT GGT GAA GCC AAG ACC GCT GTC GAG AAC TAC CTT ATC	450

GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC GAC GTG 90
 Ala Val Ala Leu Ile Gly Phe Gly Thr Val Ala Gly Ser Val

TAC CAC GAC GGT GCC TGT CCC GAA GTC AAG CCA GTC GAC AAC TTC 135
 Tyr His Asp Gly Ala Cys Pro Glu Val Lys Pro Val Asp Asn Phe
 5 10 15

5 GAC TGG TCC CAG TAC CAT GGT AAA TGG TGG GAA GTC GCC AAA TAC 180
 Asp Trp Ser Gln Tyr His Gly Lys Trp Trp Glu Val Ala Lys Tyr
 20 25 30

10 CCC AAC TCA GTT GAG AAG TAC GGA AAG TGC GGA TGG GCT GAG TAC 225
 Pro Asn Ser Val Glu Lys Tyr Gly Lys Cys Gly Trp Ala Glu Tyr
 35 40 45

ACT CCT GAA GGC AAG AGT GTC AAA GTT TCG AAC TAC CAC GTA ATC 270
 15 Thr Pro Glu Gly Lys Ser Val Lys Val Ser Asn Tyr His Val Ile
 50 55 60

CAC GGC AAG GAA TAC TTT ATT GAA GGA ACT GCC TAC CCA GTT GGT 315
 20 His Gly Lys Glu Tyr Phe Ile Glu Gly Thr Ala Tyr Pro Val Gly
 65 70 75

GAC TCC AAG ATT GGA AAG ATC TAC CAC AGC CTG ACT TAC GGA GGT 360
 Asp Ser Lys Ile Gly Lys Ile Tyr His Ser Leu Thr Tyr Gly Gly
 80 85 90

25 GTC ACC AAG GAG AAC GTA TTC AAC GTA CTC TCC ACT GAC AAC AAG 405
 Val Thr Lys Glu Asn Val Phe Asn Val Leu Ser Thr Asp Asn Lys
 95 100 105

AAC TAC ATC ATC GGA TAC TAC TGC AAA TAC GAC GAG GAC AAG AAG 450
 30 Asn Tyr Ile Ile Gly Tyr Tyr Cys Lys Tyr Asp Glu Asp Lys Lys
 110 115 120

GGA CAC CAA GAC TTC GTC TGG GTG CTC TCC AGA AGC ATG GTC CTT 495
 35 Gly His Gln Asp Phe Val Trp Val Leu Ser Arg Ser Met Val Leu
 125 130 135

ACT GGT GAA GCC AAG ACC GCT GTC GAG AAC TAC CTT ATC GGC TCC 540
 40 Thr Gly Glu Ala Lys Thr Ala Val Glu Asn Tyr Leu Ile Gly Ser
 140 145 150

CCA GTA GTC GAC TCC CAG AAA CTG GTA TAC AGT GAC TTC TCT GAA 585
 Pro Val Val Asp Ser Gln Lys Leu Val Tyr Ser Asp Phe Ser Glu
 155 160 165

45 GCC GCC TGC AAG GTC AAC AAT AGC AAC TGG TCT CAC CCG CAG TTC 630
 Ala Ala Cys Lys Val Asn Asn Ser Asn Trp Ser His Pro Gln Phe
 170 175 180

50 GAA AAA TAATAAGCTT CGGGAAGATT T ATG AAG AAA GGT TTT ATG 675
 Glu Lys Met Lys Lys Gly Phe Met
 -20 -15

55 TTG TTT ACT TTG TTA GCG GCG TTT TCA GGC TTT GCT CAG GCT GAT 720
 Leu Phe Thr Leu Leu Ala Ala Phe Ser Gly Phe Ala Gln Ala Asp

5 GAC GCG GCA ATT CAA CAA ACG TTA GCC AAA ATG GGC ATC AAA AGC 765
 Asp Ala Ala Ile Gln Gln Thr Leu Ala Lys Met Gly Ile Lys Ser
 5 10 15

10 AGC GAT ATT CAG CCC GCG CCT GTA GCT GGC ATG AAG ACA GTT CTG 810
 Ser Asp Ile Gln Pro Ala Pro Val Ala Gly Met Lys Thr Val Leu
 20 25 30

15 ACT AAC AGC GGC GTG TTG TAC ATC ACC GAT GAT GGT AAA CAT ATC 855
 Thr Asn Ser Gly Val Leu Tyr Ile Thr Asp Asp Gly Lys His Ile
 35 40 45

20 ATT CAG GGG CCA ATG TAT GAC GTT AGT GGC ACG GCT CCG GTC AAT 900
 Ile Gln Gly Pro Met Tyr Asp Val Ser Gly Thr Ala Pro Val Asn
 50 55 60

25 GTC ACC AAT AAG ATG CTG TTA AAG CAG TTG AAT GCG CTT GAA AAA 945
 Val Thr Asn Lys Met Leu Leu Lys Gln Leu Asn Ala Leu Glu Lys
 65 70 75

30 GAG ATG ATC GTT TAT AAA GCG CCG CAG GAA AAA CAC GTC ATC ACC 990
 Glu Met Ile Val Tyr Lys Ala Pro Gln Glu Lys His Val Ile Thr
 80 85 90

35 GTG TTT ACT GAT ATT ACC TGT GGT TAC TGC CAC AAA CTG CAT GAG 1035
 Val Phe Thr Asp Ile Thr Cys Gly Tyr Cys His Lys Leu His Glu
 95 100 105

40 CAA ATG GCA GAC TAC AAC GCG CTG GGG ATC ACC GTG CGT TAT CTT 1080
 Gln Met Ala Asp Tyr Asn Ala Leu Gly Ile Thr Val Arg Tyr Leu
 110 115 120

45 GCT TTC CCG CGC CAG GGG CTG GAC AGC GAT GCA GAG AAA GAA ATG 1125
 Ala Phe Pro Arg Gln Gly Leu Asp Ser Asp Ala Glu Lys Glu Met
 125 130 135

50 AAA GCT ATC TGG TGT GCG AAA GAT AAA AAC AAA GCG TTT GAT GAT 1170
 Lys Ala Ile Trp Cys Ala Lys Asp Lys Asn Lys Ala Phe Asp Asp
 140 145 150

55 GTG ATG GCA GGT AAA AGC GTC GCA CCA GCC AGT TGC GAC GTG GAT 1215
 Val Met Ala Gly Lys Ser Val Ala Pro Ala Ser Cys Asp Val Asp
 155 160 165

60 ATT GCC GAC CAT TAC GCA CTT GGC GTC CAG CTT GGC GTT AGC GGT 1260
 Ile Ala Asp His Tyr Ala Leu Gly Val Gln Leu Gly Val Ser Gly
 170 175 180

65 ACT CCG GCA GTT GTG CTG AGC AAT GGC ACA CTT GTT CCG GGT TAC 1305
 Thr Pro Ala Val Val Leu Ser Asn Gly Thr Leu Val Pro Gly Tyr
 185 190 195

70 CAG CCG CCG AAA GAG ATG AAA GAA TTC CTC GAC GAA CAC CAA AAA 1350
 Gln Pro Pro Lys Glu Met Lys Glu Phe Leu Asp Glu His Gln Lys

ATG ACC AGC GGT AAA TAATTCGCGT AGCTT
Met Thr Ser Gly Lys
215

1380

5

<210> 17

<211> 2009 bases pairs

10 <212> DNA

<213> fragment of plasmid PBBP27

<220>

<221> sig_peptide

15 <222> (23)...(85)

<220>

<221> mat_peptide

<222> (86)...(1999)

20 <223> fusion protein of alkaline phosphatase, linker peptide Pro-Pro-Ser-Ala, mutein DigA16 and Strep-Tag II

<220>

<221> CDS

25 <222> (86)...(1435)

<223> mature part of alkaline phosphatase

<220>

<221> CDS

30 <222> (1436)...(1447)

<223> linker peptide Pro-Pro-Ser-Ala

<220>

<221> CDS

35 <222> (1448)...(1969)

<223> mutein DigA16

<220>

<222> (1970)...(1999)

<223> Strep-Tag II affinity tag

<400> 17

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Val Lys Gln Ser Thr Ile Ala Leu
-21 -20 -15

10 GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAA GCC CGG ACA 91
Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Arg Thr
-10 -5 -1 1

15 CCA GAA ATG CCT GTT CTG GAA AAC CGG GCT GCT CAG GGC GAT ATT 136
Pro Glu Met Pro Val Leu Glu Asn Arg Ala Ala Gln Gly Asp Ile
5 10 15

20 ACT GCA CCC GGC GGT GCT CGC CGT TTA ACG GGT GAT CAG ACT GCC 181
Thr Ala Pro Gly Gly Ala Arg Arg Leu Thr Gly Asp Gln Thr Ala
20 25 30

25 GCT CTG CGT GAT TCT CTT AGC GAT AAA CCT GCA AAA AAT ATT ATT 226
Ala Leu Arg Asp Ser Leu Ser Asp Lys Pro Ala Lys Asn Ile Ile
35 40 45

30 TTG CTG ATT GGC GAT GGG ATG GGG GAC TCG GAA ATT ACT GCC GCA 271
Leu Leu Ile Gly Asp Gly Met Gly Asp Ser Glu Ile Thr Ala Ala
50 55 60

35 CGT AAT TAT GCC GAA GGT GCG GGC GGC TTT TTT AAA GGT ATA GAT 316
Arg Asn Tyr Ala Glu Gly Ala Gly Gly Phe Phe Lys Gly Ile Asp
65 70 75

40 GCC TTA CCG CTT ACC GGG CAA TAC ACT CAC TAT GCG CTG AAT AAA 361
Ala Leu Pro Leu Thr Gly Gln Tyr Thr His Tyr Ala Leu Asn Lys
80 85 90

45 AAA ACC GGC AAA CCG GAC TAC GTC ACC GAC TCG GCT GCA TCA GCA 406
Lys Thr Gly Lys Pro Asp Tyr Val Thr Asp Ser Ala Ala Ser Ala
95 100 105

50 ACC GCC TGG TCA ACC GGT GTC AAA ACC TAT AAC GGC GCG CTG GGC 451
Thr Ala Trp Ser Thr Gly Val Lys Thr Tyr Asn Gly Ala Leu Gly
110 115 120

55 GTC GAT ATT CAC GAA AAA GAT CAC CCA ACG ATT CTG GAA ATG GCA 496
Val Asp Ile His Glu Lys Asp His Pro Thr Ile Leu Glu Met Ala
125 130 135

60 AAA GCC GCA GGT CTG GCG ACC GGT AAC GTT TCT ACC GCA GAG TTG 541
Lys Ala Ala Gly Leu Ala Thr Gly Asn Val Ser Thr Ala Glu Leu
140 145 150

	Gln Asp	Ala Thr	Pro Ala	Ala Ala	Leu Val	Ala His	Val Thr	Ser Arg	
	155				160			165	
5	AAA TGC	TAC GGT	CCG AGC	GCG ACC	AGT GAA	AAA TGT	CCG GGT	AAC 631	
	Lys Cys	Tyr Gly	Pro Ser	Ala Thr	Ser Glu	Lys Cys	Pro Gly	Asn 180	
	170				175				
10	GCT CTG	GAA AAA	GGC GGA	AAA GGA	TCG ATT	ACC GAA	CAG CTG	CTT 676	
	Ala Leu	Glu Lys	Gly Gly	Lys Gly	Ser Ile	Thr Glu	Gln Leu	Leu 195	
	185				190				
15	AAC GCT	CGT GCC	GAC GTT	ACG CTT	GGC GGC	GGC GCA	AAA ACC	TTT 721	
	Asn Ala	Arg Ala	Asp Val	Thr Leu	Gly Gly	Gly Ala	Lys Thr	Phe 210	
	200				205				
	GCT GAA	ACG GCA	ACC GCT	GGT GAA	TGG CAG	GGA AAA	ACG CTG	CGT 766	
	Ala Glu	Thr Ala	Thr Ala	Gly Glu	Trp Gln	Gly Lys	Thr Leu	Arg 225	
	215				220				
20	GAA CAG	GCA CAG	GCG CGT	GGT TAT	CAG TTG	GTG AGC	GAT GCT	GCC 811	
	Glu Gln	Ala Gln	Ala Arg	Gly Tyr	Gln Leu	Val Ser	Asp Ala	Ala 240	
	230				235				
25	TCA CTG	AAT TCG	GTG ACG	GAA GCG	AAT CAG	CAA AAA	CCC CTG	CTT 856	
	Ser Leu	Asn Ser	Val Thr	Glu Ala	Asn Gln	Gln Lys	Pro Leu	Leu 255	
	245				250				
30	GGC CTG	TTT GCT	GAC GGC	AAT ATG	CCA GTG	CGC TGG	CTA GGA	CCG 901	
	Gly Leu	Phe Ala	Asp Gly	Asn Met	Pro Val	Arg Trp	Leu Gly	Pro 270	
	260				265				
35	AAA GCA	ACG TAC	CAT GGC	AAT ATC	GAT AAG	CCC GCA	GTC ACC	TGT 946	
	Lys Ala	Thr Tyr	His Gly	Asn Ile	Asp Lys	Pro Ala	Val Thr	Cys 285	
	275				280				
	ACG CCA	AAT CCG	CAA CGT	AAT GAC	AGT GTA	CCA ACC	CTG GCG	CAG 991	
	Thr Pro	Asn Pro	Gln Arg	Asn Asp	Ser Val	Pro Thr	Leu Ala	Gln 300	
	290				295				
40	ATG ACC	GAC AAA	GCC ATT	GAA TTG	TTG AGT	AAA AAT	GAG AAA	GGC 1036	
	Met Thr	Asp Lys	Ala Ile	Glu Leu	Leu Ser	Lys Asn	Glu Lys	Gly 315	
	305				310				
45	TTT TTC	CTG CAA	GTT GAA	GGT GCG	TCA ATC	GAT AAA	CAG GAT	CAT 1081	
	Phe Phe	Leu Gln	Val Glu	Gly Ala	Ser Ile	Asp Lys	Gln Asp	His 330	
	320				325				
50	GCT GCG	AAT CCT	TGT GGG	CAA ATT	GGC GAG	ACG GTC	GAT CTC	GAT 1126	
	Ala Ala	Asn Pro	Cys Gly	Gln Ile	Gly Glu	Thr Val	Asp Leu	Asp 345	
	335				340				
55	GAA GCC	GTA CAA	CGG GCG	CTG GAA	TTC GCT	AAA AAG	GAG GGT	AAC 1171	
	Glu Ala	Val Gln	Arg Ala	Leu Glu	Phe Ala	Lys Lys	Glu Gly	Asn 360	
	350				355				

	Thr	Leu	Val	Ile	Val	Thr	Ala	Asp	His	Ala	His	Ala	Ser	Gln	Ile	
			365					370					375			
5	GTT	GCG	CCG	GAT	ACC	AAA	GCT	CCG	GGC	CTC	ACC	CAG	GCG	CTA	AAT	1261
	Val	Ala	Pro	Asp	Thr	Lys	Ala	Pro	Gly	Leu	Thr	Gln	Ala	Leu	Asn	
			380					385					390			
10	ACC	AAA	GAT	GGC	GCA	GTG	ATG	GTG	ATG	AGT	TAC	GGG	AAC	TCC	GAA	1306
	Thr	Lys	Asp	Gly	Ala	Val	Met	Val	Met	Ser	Tyr	Gly	Asn	Ser	Glu	
			395					400					405			
15	GAG	GAT	TCA	CAA	GAA	CAT	ACC	GGC	AGT	CAG	TTG	CGT	ATT	GCG	GCG	1351
	Glu	Asp	Ser	Gln	Glu	His	Thr	Gly	Ser	Gln	Leu	Arg	Ile	Ala	Ala	
			410					415					420			
20	TAT	GGC	CCG	CAT	GCC	GCC	AAT	GTT	GTT	GGA	CTG	ACC	GAC	CAG	ACC	1396
	Tyr	Gly	Pro	His	Ala	Ala	Asn	Val	Val	Gly	Leu	Thr	Asp	Gln	Thr	
			425					430					435			
25	GAT	CTC	TTC	TAC	ACC	ATG	AAA	GCC	GCT	CTG	GGG	CTG	AAA	CCG	CCT	1441
	Asp	Leu	Phe	Tyr	Thr	Met	Lys	Ala	Ala	Leu	Gly	Leu	Lys	Pro	Pro	
			440					445					450			
30	AGC	GCT	GAC	GTG	TAC	CAC	GAC	GGT	GCC	TGT	CCC	GAA	GTC	AAG	CCA	1486
	Ser	Ala	Asp	Val	Tyr	His	Asp	Gly	Ala	Cys	Pro	Glu	Val	Lys	Pro	
			455					460					465			
35	GTC	GAC	AAC	TTC	GAC	TGG	TCC	CAG	TAC	CAT	GGT	AAA	TGG	TGG	CAG	1531
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22

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Claims

1. A polypeptide selected from muteins of the bilin-binding protein, characterized in that it

5 (a) is able to bind digoxigenin or digoxigenin conjugates,

(b) does not bind ouabain, testosterone and 4-aminofluorescein and

(c) has an amino acid substitution at at least one
10 of the sequence positions 28, 31, 34, 35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125, and 127 of the bilin-binding protein.

2. The polypeptide according to claim 1,
15 characterized in that the dissociation constant of the complex with digoxigenin is 100 nM or less.

3. The polypeptide according to claim 1 or 2, characterized in that it carries, in comparison with
20 the bilin-binding protein, at least one of the amino acid substitutions selected from Glu(28)->Gln, Lys(31)->Ala, Asn(34)->Asp, Ser(35)->His, Val(36)->Ile, Glu(37)->Thr, Asn(58)->Arg, His(60)->Ser, Ile(69)->Ser, Leu(88)->Tyr, Tyr(90)->Ile, Lys(95)->Gln, Asn(97)->Gly,
25 Tyr(114)->Phe, Lys(116)->Ser, Gln(125)->Met, and Phe(127)->Leu.

4. The polypeptide according to claim 3, characterized in that it has the amino acid sequence
30 depicted as SEQ ID NO:15.

5. The polypeptide as claimed in one or more of claims 1 to 4, characterized in that it carries at least one label group, selected from enzymatic label,
35 radioactive label, fluorescent label, chromophoric label, (bio)luminescent label or label containing haptens, biotin, metal complexes, metals or colloidal

6. Fusion proteins of polypeptides according to one or more of claims 1 to 5, characterized in that an enzyme, another protein or a protein domain, a signal sequence and/or an affinity peptide is fused to the amino terminus of the polypeptide in an operable manner.

7. Fusion proteins of polypeptides according to one or more of claims 1 to 6, characterized in that an enzyme, another protein or a protein domain, a targeting sequence and/or an affinity peptide is fused to the carboxy terminus of the polypeptide in an operable manner.

8. A nucleic acid, characterized in that it comprises a sequence coding for a mutein or a fusion protein of a mutein of the bilin-binding protein according to one or more of claims 1 to 7.

9. The nucleic acid according to claim 8, characterized in that it comprises the nucleotide sequence according to SEQ ID NO:15 or another nucleotide sequence encoding the polypeptide according to SEQ ID NO:15.

10. A method for producing digoxigenin-binding muteins of the bilin-binding protein, which comprises the following steps:

(a) subjecting the bilin-binding protein to random mutagenesis at at least one of the sequence positions 28, 31, 34, 35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125, and 127,

(b) enriching resulting muteins with binding affinity for the digoxigenin group by selection and isolating said muteins,

(c) subjecting the muteins obtained in step (b) to another random mutagenesis at at least one of the

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(d) again enriching the resulting muteins by selection and isolating said muteins.

11. The method according to claim 10, wherein in
5 step (b) selection is carried out by competitive enrichment.

12. The method according to claim 11, wherein
free digoxigenin or digitoxigenin is used for
10 competitive enrichment.

13. The method according to any of claims 10 to
12, wherein the enrichment in step (d) is carried out
by forming a complex of the muteins with the
15 digoxigenin group and subsequently dissociating the
complex.

14. The method according to claim 13, wherein the
dissociation of the complex of mutein and digoxigenin
20 group is carried out in acidic or basic medium milieu.

15. A method for preparing a mutein or a fusion
protein of a mutein of the bilin-binding protein
according to one or more of claims 1 to 7 or for
25 preparing a mutein which is obtainable according to a
method according to one or more of claims 10 to 14,
characterized in that the nucleic acid coding for the
mutein or the fusion protein of a mutein of the bilin-
binding protein is expressed in a bacterial or
30 eukaryotic host cell and the polypeptide is obtained
from the cell or the culture supernatant.

15. The use of a mutein or a fusion protein of a
mutein of the bilin-binding protein according to one or
35 more of claims 1 to 7 or of a mutein which is
obtainable according to a method according to one or
more of claims 10 to 14 for binding, detecting,
determining, immobilizing or removing digoxigenin or

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(d) again enriching the resulting muteins by selection and isolating said muteins.

11. The method according to claim 10, wherein in
5 step (b) selection is carried out by competitive enrichment.

12. The method according to claim 11, wherein
free digoxigenin or digitoxigenin is used for
10 competitive enrichment.

13. The method according to any of claims 10 to
12, wherein the enrichment in step (d) is carried out
by forming a complex of the muteins with the
15 digoxigenin group and subsequently dissociating the
complex.

14. The method according to claim 13, wherein the
dissociation of the complex of mutein and digoxigenin
20 group is carried out in acidic or basic medium milieu.

15. A method for preparing a mutein or a fusion
protein of a mutein of the bilin-binding protein
according to one or more of claims 1 to 7 or for
25 preparing a mutein which is obtainable according to a
method according to one or more of claims 10 to 14,
characterized in that the nucleic acid coding for the
mutein or the fusion protein of a mutein of the bilin-
binding protein is expressed in a bacterial or
30 eukaryotic host cell and the polypeptide is obtained
from the cell or the culture supernatant.

15. The use of a mutein or a fusion protein of a
mutein of the bilin-binding protein according to one or
35 more of claims 1 to 7 or of a mutein which is
obtainable according to a method according to one or
more of claims 10 to 14 for binding, detecting,
determining, immobilizing or removing digoxigenin or

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carbohydrates, other biological or synthetic macromolecules or low molecular weight chemical compounds.

- 5 17. A method for detecting the digoxigenin group, wherein a mutein of the bilin-binding protein or a fusion protein of a mutein of the bilin-binding protein according to one or more of claims 1 to 7 or a mutein which is obtainable according to a method according to
- 10 one or more of claims 10 to 14 is brought into contact with digoxigenin or with conjugates of digoxigenin under conditions suitable for effecting binding of the mutein to the digoxigenin group, and the mutein or the fusion protein of the mutein is determined.

15

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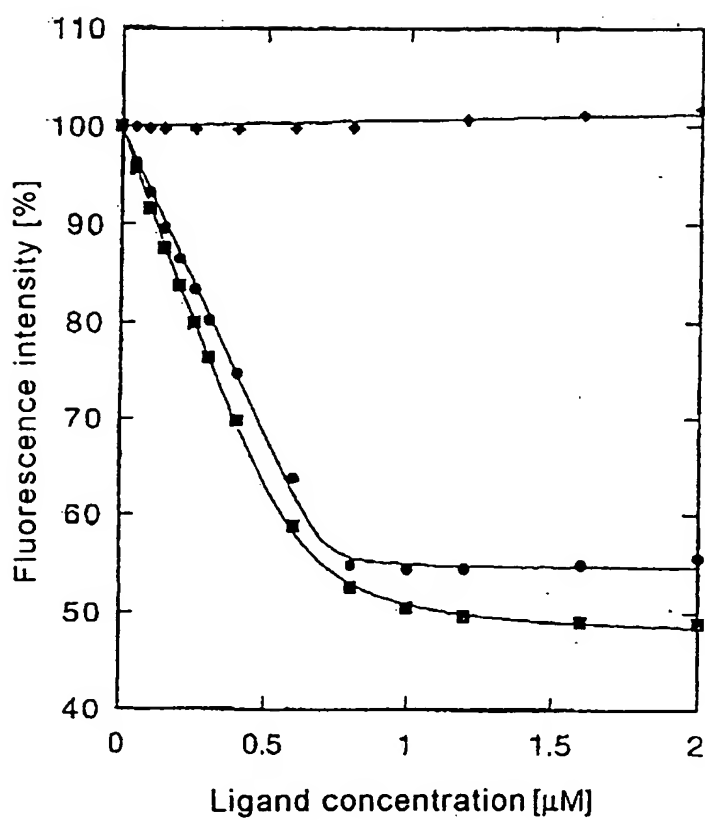


Figure 1

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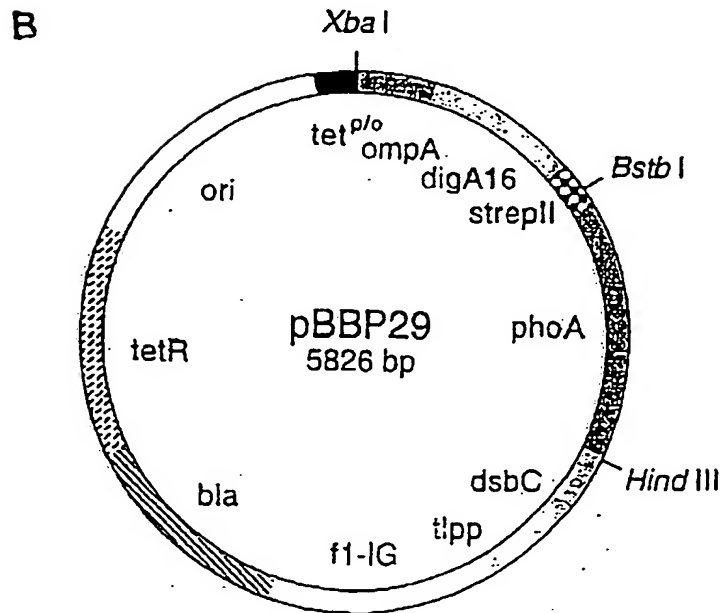
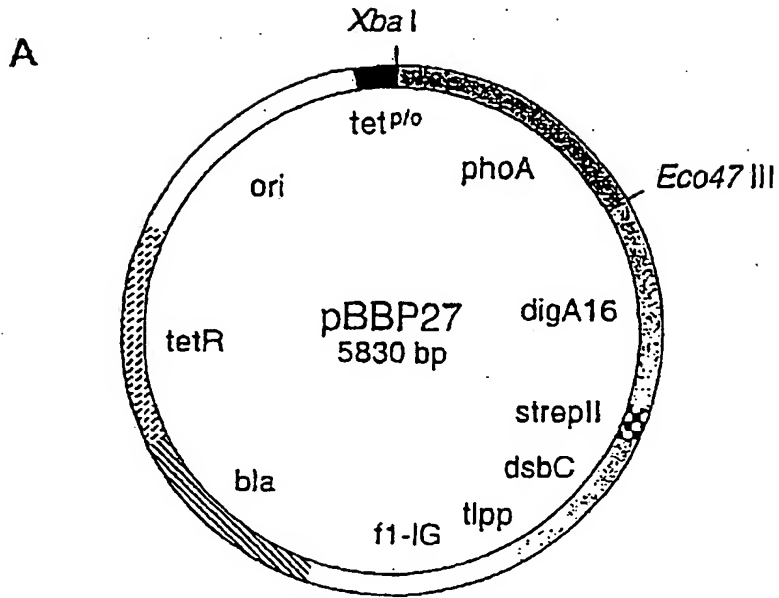


Figure 2

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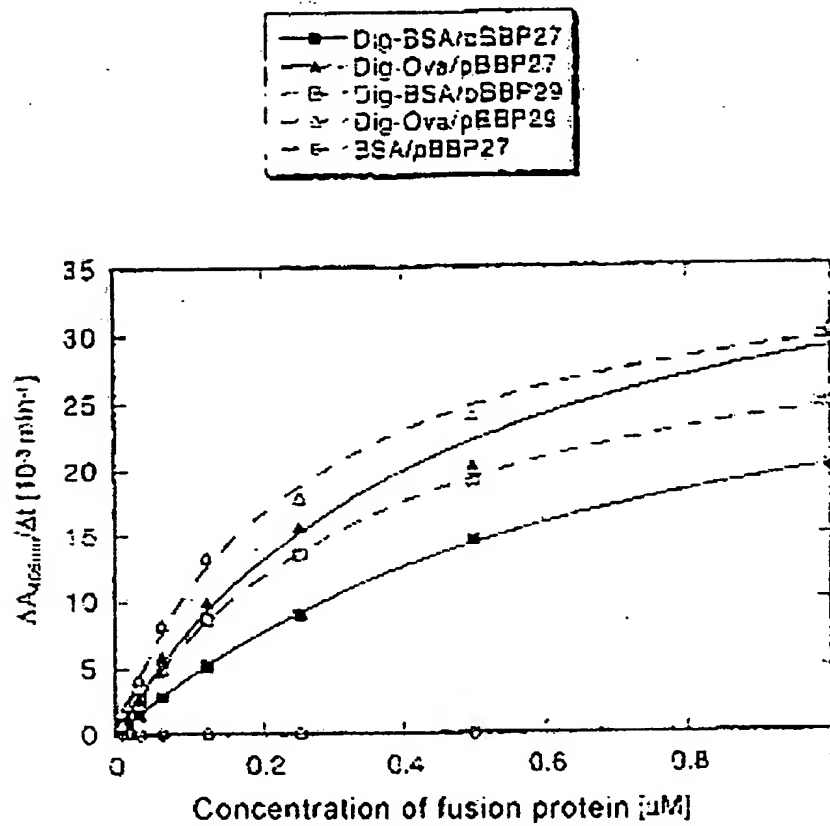


Figure 3

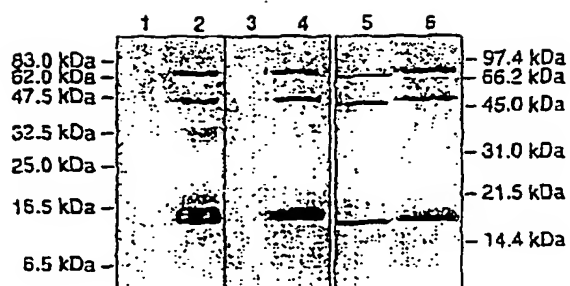
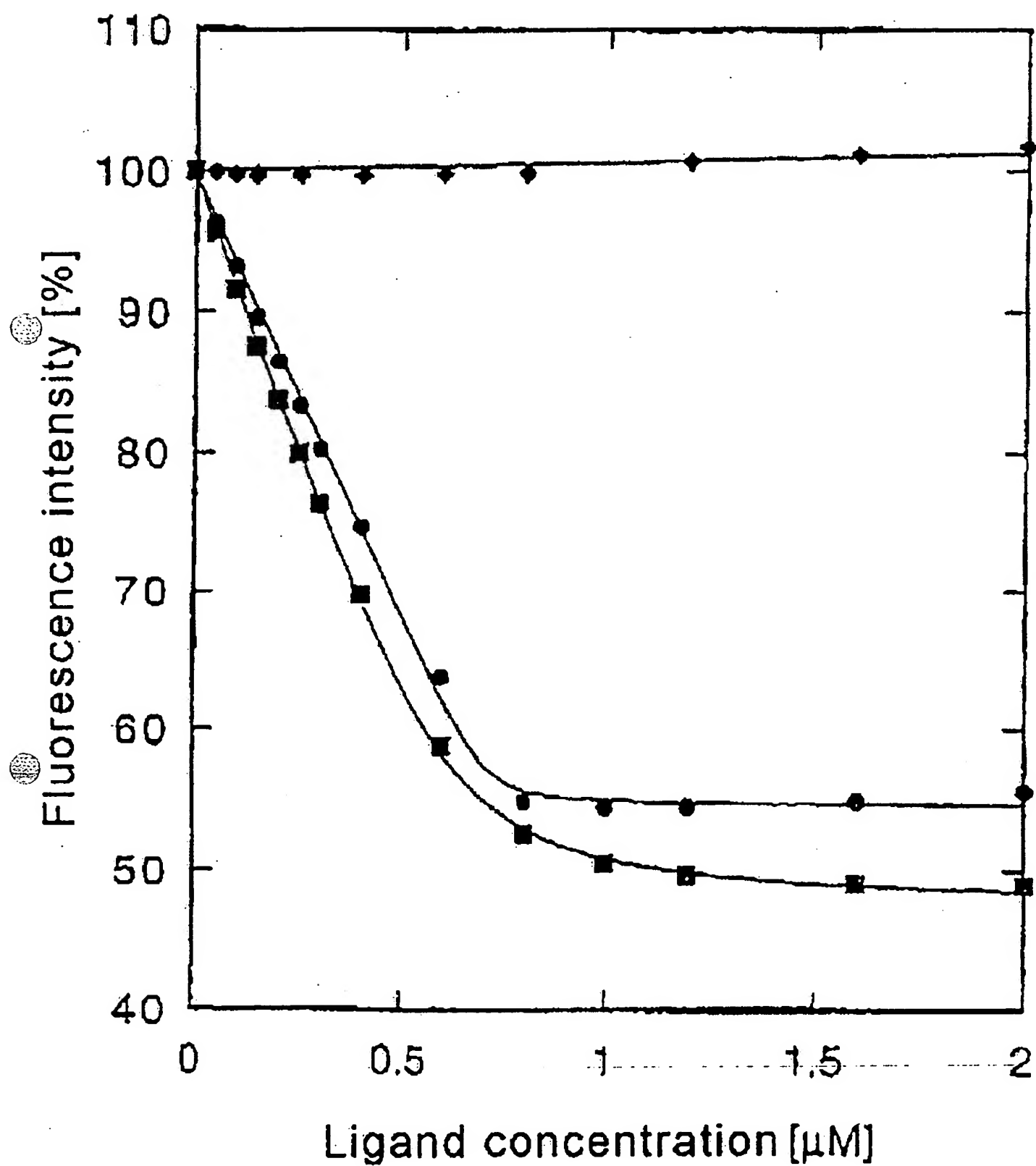


Figure 4



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